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The O-methyl Ribosyl Constituents and Chain Termini
of Escherichia coli RNA

by



Jack Loran Nichols

A Thesis

Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements for the Degree
of Doctor of Philosophy

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The O-methyl Ribosyl Constituents and Chain Termini of Escherichia coli RNA", submitted by Jack Loran Nichols in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The thesis concerns an examination of minor nucleotide components in E. coli RNA. As recently as ten years ago, it appeared that the four major ribonucleotides (adenylate, guanylate, cytidylate, uridylate) were the sole components of RNA, but it is now well-established that there are numerous minor components in the RNA from animal, plant and microbial cells. An individual minor component can account for anywhere between 0.025% and 10 % of the component nucleotides in an RNA specimen. For instance, a minor component discovered in this investigation, $N^4,2'\text{-}\underline{O}$ -dimethyl cytidylate, accounts for just 0.07 mole % of the component nucleotides in the RNA specimen from which it can be isolated. The possible significance of such a minor component becomes apparent when it is realized that this trace amount of $N^4,2'\text{-}\underline{O}$ -dimethyl cytidylate in the 16S RNA from E. coli, corresponds to an average of one such nucleotide per polynucleotide chain. A polynucleotide chain in 16S RNA contains about 1300 nucleotide residues, and the polymer is broadly characterized by the redundant occurrence of a small number of monomer units : the four major nucleotides. However, pending a uniform distribution of the minor component among the polynucleotides in 16S RNA, at only one position in the linear array of more than 1000 nucleotides per chain, there is a unique component : $N^4,2'\text{-}\underline{O}$ -dimethyl cytidylate.

The very fact of its occurrence makes the minor component a potentially useful reference point for nucleotide-sequence studies of the polynucleotide. Additionally, it seems not improbable, that the singular occurrence of this unique nucleotide at one position in a polynucleotide chain may reflect a unique structural or functional role for it in the metabolic involvements of the polynucleotide.

At the outset of this investigation, it was well-established that 2'-O-methyl nucleotides were minor components of ribosomal RNA from animal and plant cells, but nothing whatever was known about their biogenesis. Bacterial systems were known to be singularly useful for studies of the biogenesis of other methyl-substituted components in RNA, and since preliminary evidence had indicated that 2'-O-methyl nucleotides were probably present in E. coli ribosomal RNA, the present investigation was undertaken in order to identify the 2'-O-methyl nucleotides in E. coli ribosomal RNA, and to examine their in vivo and in vitro biogenesis. It is hoped that these studies will contribute to exploiting the potential utility of these minor components for structural studies of ribosomal RNA, and that the findings will be of value in future investigations into the structural and functional significance of the 2'-O-methyl nucleotide components in ribosomal RNA.

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ABBREVIATIONS

N	ribonucleoside [eg. adenosine (A), guanosine (G), cytidine (C), or uridine (U)]
Np	nucleoside 2'(3')-monophosphate
pN	nucleoside 5'-monophosphate
pNp	nucleoside 2'(3'),5'-diphosphate
pN>p, pNcp	nucleoside 2',3'-cyclic 5'-diphosphate
Nx	2'- <u>O</u> -methyl ribonucleoside [eg. 2'- <u>O</u> -methyl adenosine (Ax), 2'- <u>O</u> -methyl guanosine (Gx), 2'- <u>O</u> -methyl cytidine (Cx), 2'- <u>O</u> -methyl uridine (Ux)]
ψ	pseudouridine (5-ribosyluracil)
rRNA	16 → 18S + 23 → 32S ribonucleates insoluble in molar sodium chloride solution at 0°; derived mainly from the particulate ribosomes of cellular cytoplasm, nucleoli, and mitochondria, but probably containing small amounts of polydisperse messenger RNA (mRNA)
sRNA	4S ribonucleates, soluble in molar sodium chloride solution at 0°; derived mainly from the soluble fraction of cells, but probably containing small amounts of 5 → 7S RNA from ribosomes
CM-rRNA	rRNA prepared from cells grown in the presence of chloramphenicol

MS-rRNA	rRNA prepared from cells of <u>E. coli</u> K ₁₂ W6 (RC ^{rel.} , meth. ⁻ , biotin ⁻ , λ ⁺ , F ⁺) grown in a methionine-deficient medium
(<u>methyl</u> - ¹⁴ C)-rRNA	rRNA prepared from cells grown in the presence of (<u>methyl</u> - ¹⁴ C)-L-methionine
(<u>methyl</u> - ¹⁴ C, ³² P)-rRNA	rRNA prepared from cells grown in a medium containing (<u>methyl</u> - ¹⁴ C)-L-methionine and ³² P-inorganic phosphate
(<u>methyl</u> - ¹⁴ C, ³² P)-CM-rRNA	rRNA prepared from cells grown in a medium containing (<u>methyl</u> - ¹⁴ C)-L-methionine, ³² P-inorganic phosphate, and chloramphenicol
CM-particles	chloramphenicol particles; 18S + 25S ribonucleoprotein particles prepared from <u>E. coli</u> B grown in the presence of chloramphenicol
MS-particles	"methionine-starvation" particles; "relaxed-control" particles; 20S + 23S ribonucleoprotein particles prepared from <u>E. coli</u> K ₁₂ W6 (RC ^{rel.} , meth. ⁻ , biotin ⁻ , λ ⁺ , F ⁺) grown in a methionine-deficient medium
DEAE-cellulose	diethylaminoethyl-cellulose
TRIS	tris (hydroxymethyl) amine
A _{260mμ} (A _{280mμ})	absorbance at 260 (280) millimicrons

The N-heterocycle* and N-glycosyl** radicals, which comprise the nucleoside constituents of nucleates, are contracted to heterocycle and glycosyl, respectively, in the thesis.

* Purinyl and pyrimidinyl radicals.

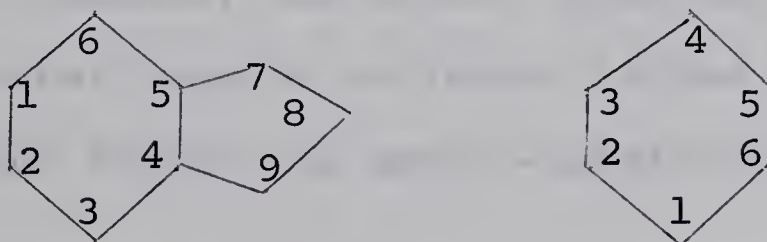
** O-heterocycles : ribosyl, deoxyribosyl, 2'-O-methyl ribosyl radicals.

The symbols used as abbreviations for methyl-substituted nucleotides can be explained most simply by reference to a series of methyl-substituted cytidines. To indicate methyl-substitution of a carbon atom in the heterocycle, as, for instance, in the case of 5-methyl cytidine, the symbol for cytidine (C) is prefixed by the symbols "5Me", ie., 5MeC. To indicate methyl-substitution of a nitrogen atom in the heterocycle, as, for instance, in the case of 3-methyl cytidine, the symbol for cytidine (C) is prefixed by the symbols "3Me", ie., 3MeC. To indicate methyl-substitution of the exocyclic nitrogen atom at C-4, as, for instance, in the case of 1-ribosyl-2-keto-4-methylamino-pyrimidine, the symbol for cytidine (C) is prefixed by the symbols "N⁴Me", ie., N⁴MeC.

In referring to 5'-nucleotides of these compounds, the symbols used to designate the position of methyl substitution will be placed between the symbols for "phosphate" (p) and "nucleoside" (N), ie., pN⁴MeC, except in rare instances, where, for purposes of tabulation, it is useful to use the designation N⁴MepC (cf. Table 2).

The term "peak" is used in the accepted sense with reference to elution profiles, and the terms "spot" and "band" are used in the accepted sense with reference to paper chromatograms and electrophoretograms, without the use of quotation marks in the body of the thesis.

The convention of numbering the positions in purine and pyrimidine compounds is indicated below.



PREFACE

Miescher reported the isolation from pus cells of an unusual phosphorus-containing material, nuclein, in 1871, and by about 1900, this fundamental observation had led to the isolation of protein-free nucleates from animal, plant, and microbial cells (eg. thymus, wheat embryo, yeast) (see Allen, 1962). During this same period (ca. 1870-1900), it was found that acid hydrolyzates of nucleates contained five "major" N-heterocycles, which had not been previously characterized, and which were identified largely through the efforts of Fischer and his co-workers (see Levene and Bass, 1931). Thus, by about 1900, two broad classes of nucleates were distinguished from one another by the nature of the N-heterocycles formed upon vigorous acid hydrolysis: one type of nucleate yielded adenine, guanine (purine derivatives), cytosine, and uracil (pyrimidine derivatives), while the other type of nucleate yielded adenine, guanine, cytosine, and thymine (a methyl-substituted derivative of uracil).

In 1909, Levene and Jacobs reported that mild acid hydrolysis of the uracil-containing nucleate gave rise to a hitherto unknown sugar, which was identified as D-ribose. In 1929, Levene and Mori reported that very mild acid hydrolysis of the thymine-containing nucleate gave rise to a

sugar, which was identified as D-2-deoxyribose, also hitherto unknown. Thus, by about 1930, it became possible to distinguish the two broad classes of nucleate in terms of their component glycosyl radicals: the ribose-containing nucleates (Ribonucleates, RNA), and the deoxyribose-containing nucleates (Deoxyribonucleates, DNA). These early studies, using the classical methods of organic chemistry were far from quantitative, as witness, the fact that Levene and Mori recovered D-2-deoxyribose from DNA in only 2% yield.

During the ensuing twenty years (ca. 1930-1950), the basic structural units of the nucleates, the nucleosides, were characterized as being β -D-N-glycofuranosyl derivatives of the N-heterocycles, and sites of attachment between the N-heterocycles and their N-glycosyl radicals were established (see Michelson, 1963). By the early 1950's, it had been established that the nucleates were probably linear polymers comprised of nucleosides linked together by internucleoside 3'-5' phosphodiester bonds (Brown and Todd, 1952; Markham and Smith, 1952; Khym, Doherty, Volkin, and Cohn, 1953).

With the advent of new and powerful techniques for resolving mixtures of low-molecular weight compounds (paper chromatography, paper electrophoresis, polystyrene ion-exchange chromatography) (see Chargaff and Davidson, 1955), it became possible in the 1940's and 1950's, to perform quantitative studies of the products formed by hydrolysis of the nucleates. A search for small amounts of "minor" components

in nucleate-hydrolyzates led to several significant advances: (i) Hotchkiss discovered a "minor" methyl-substituted N-heterocycle (5-methyl cytosine) in hydrolyzates of thymus DNA, (ii) Dunn and his co-workers discovered a host of methyl-substituted N-heterocycles in hydrolyzates of animal, plant, and microbial RNA, (iii) Smith and Dunn discovered a methyl-substituted glycosyl (2'-O-methyl ribosyl) in hydrolyzates of animal, plant, and microbial RNA, and (iv) Allen and his co-workers, and Cohn, independently discovered a "minor" component which contained a C-glycosyl rather than a N-glycosyl bond (5-ribosyl uracil or pseudouridine), in hydrolyzates of yeast RNA.

In recent years, the introduction of cellulose ion-exchange chromatography (Sober and Petersen, 1956), dextran-gel chromatography and polyacrylamide-gel electrophoresis, has made it possible to resolve complex mixtures of high molecular weight compounds, such as the nucleates, even though there are only slight differences of molecular weight among the different species of polynucleotide present in the mixture. This development has led to the recognition that not only the ribonucleates from the soluble fraction of cells (sRNA), but probably, also, the ribonucleates from the subcellular particulates (rRNA) are substantially heterogeneous (Peacock and Dingman, 1967). Thus, in a quantitative sense, the term "minor" component has a diffuse meaning, since for example, isopentenyl

adenine accounts for ca. 0.01 mole % of the N-heterocycles in yeast sRNA (heterogeneous) (Hall, 1966), but it accounts for ca. 1.3 mole % of the N-heterocycles in yeast seryl-sRNA (homogeneous) (Zachau, Dutting, and Feldman, 1966).

A wide variety of minor components is now known to be present in some cellular nucleates (sRNA, rRNA, DNA) and viral DNA, although none have so-far been isolated from other cellular nucleates (mRNA, 5-7S RNA) or viral RNA. These minor components will be qualitatively surveyed below, and catagorized with respect to the way in which they can be considered to derive, by simple chemical modification, from the major components of RNA and DNA.

DNA

(i) Methyl-substitution of a N-heterocycle.

The first discovery of a bona fide minor component, of any kind, in cellular nucleates, stemmed from studies by Hotchkiss (1948), who noted an ultraviolet absorbing component with the spectral properties of 5-methyl cytosine on paper chromatograms of acid hydrolyzates of DNA. It was not until 1951, however, that Wyatt reported the definitive identification of 5-methyl cytosine from the DNA of several animal and plant cells. Earlier claims to the discovery of minor components in nucleate-hydrolyzates (eg. guanine-uridylic acid, see Michelson, 1963, Introduction) were not authenticated. Another methyl-

substituted N-heterocycle, N⁶-methyl adenine, has been found to be a component of E. coli DNA (Dunn and Smith, 1955).

(ii) Hydroxymethyl-substitution of a N-heterocycle.

Wyatt and Cohen, (1953) found that 5-hydroxymethyl cytosine replaced cytosine in the DNA from several T-even E. coli-bacteriophages.

(iii) Glucosyl and Gentiobiosyl-substitution of a minor N-heterocycle.

Sinsheimer (1954) and Jesaitis (1956) showed that the DNA of T-even bacteriophages contained glucosyl radicals bonded in O-glycosyl linkage to the hydroxymethyl substituents of cytosine. Kuno and Lehman (1962) also found that gentiobiose could be attached in O-glycosyl linkage to the hydroxymethyl substituents of cytosine in the DNA of T6-E. coli bacteriophage.

RNA

(i) Carbon-Carbon bond between a N-heterocycle and glycosyl.

The first discovery of a bona fide minor component in RNA, stemmed from the studies of Davis and Allen (1957), who discovered a "fifth" nucleotide in hydrolyzates of yeast RNA. Positive identification of this component as 5-ribosyl uracil (pseudouridine) was provided by Yu and Allen (1959), Scannell, Crestfield, and Allen (1959) and Cohn (1959).

(ii) Deamination of a N-heterocycle.

Numerous early claims that hypoxanthine was a minor N-heterocycle in nucleates were subsequently relinquished, because the component probably derived by deamination of adenine during isolation. However, Hall (1963a) showed that hypoxanthine is a minor component of yeast sRNA, and it is now known that it is a component of yeast alanyl-sRNA and yeast seryl-sRNA (Holley, Apgar, Everett, Madison, Marquisee, Merrill, Penswick, and Zamir, 1965; Zachau, Dutting, and Feldmann, 1966).

(iii) Reduction of a N-heterocycle.

Madison and Holley (1965) first reported the presence of 5,6-dihydro uracil in yeast alanyl-sRNA, and it was later found that 5,6-dihydrouracil comprises nearly 10% of the N-heterocycles in yeast tyrosyl-sRNA (Madison, Everett, and Kung, 1966).

(iv) Sulphur analogue of a N-heterocycle.

Two thio-pyrimidines have been reported to be components of RNA. Lipsett (1965) reported the occurrence of 4-thio-uracil in E. coli sRNA, and Carbon et al. (1965) reported the occurrence of 2-thio-uracil in sRNA from animal and microbial cells.

(v) Methyl-substitution of a N-heterocycle.

By far the largest number of minor components in RNA fall into this category. Dunn and his co-workers introduced a new era in the analytical biochemistry of RNA,

when they discovered a multitude of methyl-substituted N-heterocycles in hydrolyzates of animal, plant, and microbial RNA. These findings provided the impetus that led to the discovery of most of the other minor components described in other categories of this present survey.

The methyl-substituted N-heterocycles have been found in the following nucleosides: 1MeA (Dunn, 1961), 2MeA (Littlefield and Dunn, 1958, 1958a), N⁶MeA (Littlefield and Dunn, 1958, 1958a), N⁶,N⁶diMeA (Littlefield and Dunn, 1958, 1958a), 1MeG (Adler et al., 1958; Smith and Dunn, 1959a), 7MeG (Dunn and Spahr, 1961), N²MeG (Adler et al., 1958; Smith and Dunn, 1959a), N²,N²diMeG (Smith and Dunn, 1959a), 5MeC (Amos and Korn, 1958; Dunn, 1959), 3MeC (Hall, 1963), 3MeU (Hall, 1963), and 5MeU (Littlefield and Dunn, 1958, 1958a). Most of these components are present in the sRNA from animal and plant cells, although fewer of them have been found in bacterial sRNA. All of the purified aminoacyl-sRNA specimens have been found to contain a significant complement of these methyl-substituted N-heterocycles, and many of them are also present in animal, plant, and microbial rRNA.

(vi) Methyl-substitution of a glycosyl.

Smith and Dunn (1959) first reported the isolation of 2'(3')-O-methylated derivatives of adenosine, guanosine, cytidine, and uridine from animal, plant, and microbial RNA.

(vii) Methyl-substitution of a minor component.

Hall (1963a) showed that 1-methyl hypoxanthine was a component of yeast sRNA, and it has since been found that 1-methyl hypoxanthine is a component of yeast alanyl-sRNA (Holley et al., 1965). Hall (1964) showed that 2'-O-methyl pseudouridine was a component of animal and microbial sRNA, and Hudson, Gray, and Lane (1965) showed it to be a component of plant sRNA.

(viii) Isopentenyl-substitution of a N-heterocycle.

N⁶-(γ , γ -dimethylallyl) adenosine has been shown to be present in yeast sRNA (Hall, 1966), and it has recently been demonstrated that it is a component of yeast seryl-sRNA (Zachau, Dutting, and Feldmann, 1966a).

(ix) Ribosyl-substitution of a glycosyl.

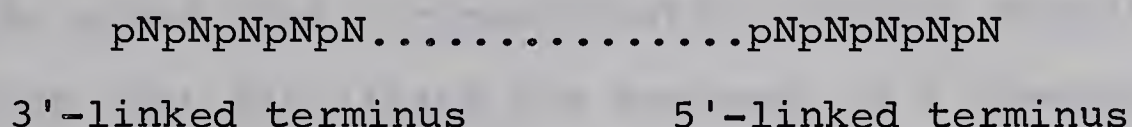
Hall (1965) has isolated from yeast sRNA a component that is believed to be 2'(3')-O-ribosyl adenosine.

(x) Acyl, Aminoacyl or Carboxy-methyl-substitution of a N-heterocycle or glycosyl.

2'(3')-O-aminoacyl adenosine has been isolated from the chain termini of sRNA (Zachau, Acs, and Lipmann, 1958). A series of compounds, N⁶-(N-formyl- α -aminoacyl) adenosines, have been isolated from hydrolyzates of yeast sRNA (Hall, 1964a; Hall and Chheda, 1965). Feldmann, Dutting, and Zachau (1966) have isolated and characterized N⁴-acetyl cytidine from yeast seryl-sRNA. Gray and Lane (1967) have isolated 5-carboxymethyl uracil from yeast and wheat embryo sRNA.

(xi) Minor components from chain termini.

Hydrolysis of RNA in 1 M NaOH for 24 hr. at room temperature (Steudel and Peiser, 1921) yields small amounts of nucleosides and nucleoside 2'(3'),5'-diphosphates from 5'-linked and 3'-linked chain termini, respectively (Markham, Matthews, and Smith, 1954).



The studies of this thesis are primarily concerned with the minor components in E. coli rRNA and sRNA, and more particularly, with those in categories (vi) and (xi).

Part I. The Alkali-Stable Dinucleotide Sequences in Escherichia coli Ribonucleates.

(i) Introduction

The studies in this part of the thesis were designed to characterize 2'-O-methyl ribose-containing, alkali-stable dinucleotide sequences in E. coli RNA. It should be noted that 2'-O-methylation confers singular properties that facilitate the recovery of 2'-O-methyl ribosyl derivatives from the remainder of the components in RNA hydrolyzates. Thus, the 2'-O-methyl substituent confers alkali-stability on the internucleoside phosphodiester bond to the 3'-position of the O-methyl nucleoside constituents of ribonucleate chains (Smith and Dunn, 1959). As a consequence, after complete alkali hydrolysis of RNA, the 2'-O-methyl nucleosides are found as part of alkali-stable dinucleotides (Smith and Dunn, 1959; Lane and Butler, 1959). These dinucleotides can be separated from the bulk of the hydrolysis products (mononucleotides) by a simple stepwise elution of mononucleotides, and then dinucleotides, from DEAE-cellulose (Singh and Lane, 1964, 1964a). Similarly, the 2'-O-methyl substituent confers resistance to snake venom 5'-nucleotidase (Honjo, Kanai, Furukawa, Mizuno, and Sanno, 1964), and as a consequence, 2'-O-methyl nucleosides can be re-

covered as 2'-O-methyl nucleoside 5'-phosphates after hydrolysis of RNA with whole snake venom (Hudson, Gray, and Lane, 1965). These 2'-O-methyl nucleoside 5'-phosphates can be separated from the bulk of the hydrolysis products (nucleosides) by passage of whole venom hydrolyzates through DEAE-cellulose, in which case, the 2'-O-methyl nucleoside 5'-phosphates are adsorbed, and the nucleosides are not adsorbed.

Smith and Dunn (1959) demonstrated that cellular RNA from a variety of animal, plant, and microbial sources, contained alkali-stable dinucleotide sequences. The alkali-stable sequences in the rRNA and sRNA from wheat embryo (Singh and Lane, 1964a; Hudson, Gray, and Lane, 1965; Lane, 1965), and more recently, those in the rRNA and sRNA from yeast (Gray and Lane, 1967), have been examined in detail, and their quantitative proportions have been measured. It was of interest to extend the analyses of wheat embryo and yeast rRNA and sRNA to rRNA and sRNA of bacterial origin.

Although the presence of several methyl-substituted nucleoside constituents in rRNA has been demonstrated (Dunn, 1959; Svensson et al., 1963; Starr and Fefferman, 1964), the larger quantity of such components in sRNA, relative to rRNA, makes data from analyses of rRNA, subject to the criticism that, these minor constituents may arise

from contamination of rRNA preparations with sRNA. This is a particularly important consideration in the present studies since a novel nucleoside, N⁴,2'-O-dimethyl cytidine, has been found as part of an alkali-stable dinucleotide sequence in E. coli rRNA. For this reason, a parallel examination of the alkali-stable dinucleotide sequences in E. coli sRNA was undertaken, in order to demonstrate that the sequence distribution of 2'-O-methyl nucleoside constituents was different in the two types of RNA. An additional degree of analytical discernment was provided by the isolation of 2'-O-methyl nucleosides as part of dinucleotides, rather than as the methylated nucleoside or mononucleotide.

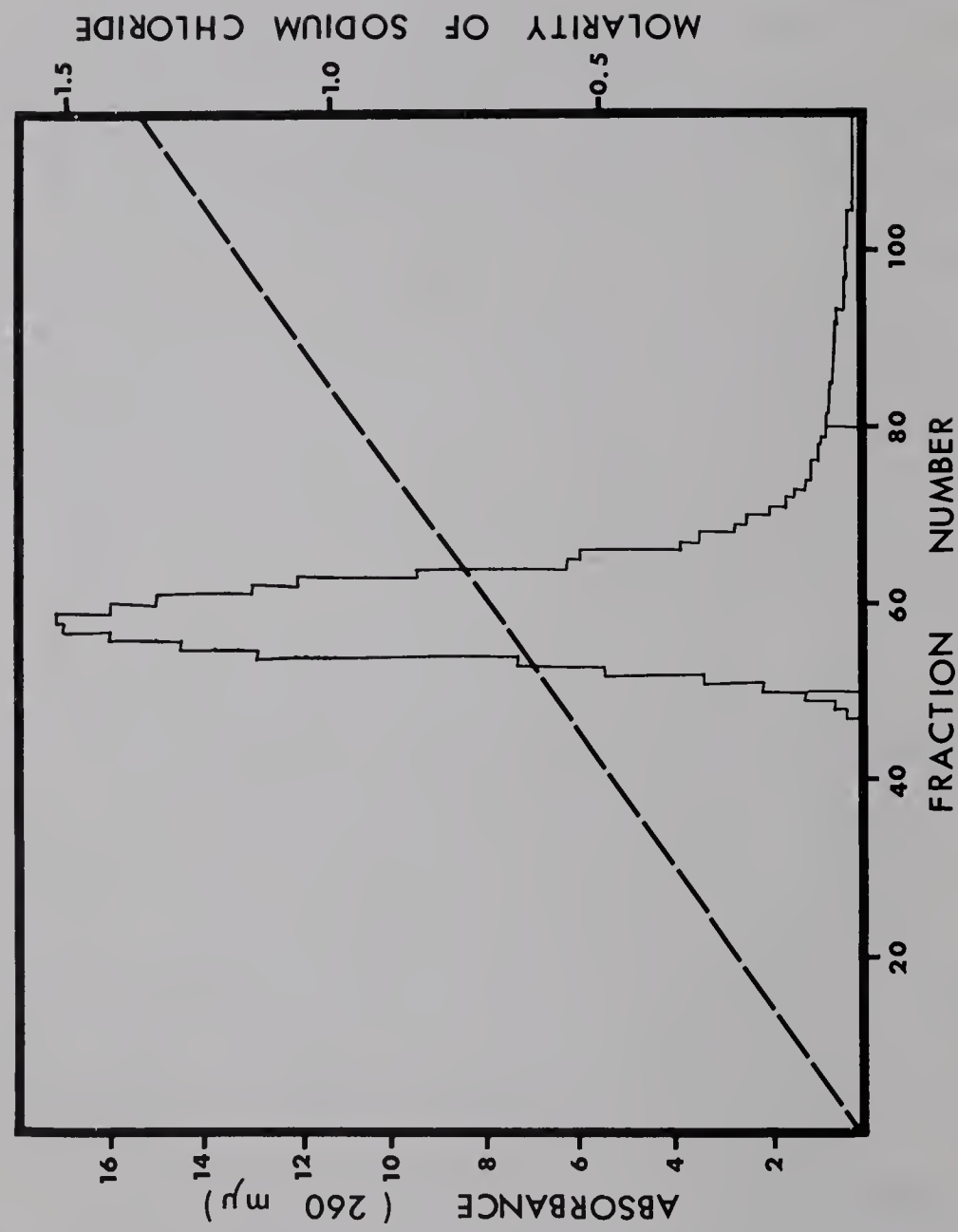
The spectral and chromatographic characterization of alkali-stable dinucleotides, was an important prerequisite to the studies in subsequent parts of this thesis, where the same sequences were identified by co-chromatography of radioactive dinucleoside monophosphates with the spectrally-characterized carrier compounds.

(ii) Materials

(1) Preparation of Large Quantities of E. coli rRNA.

Ribonucleates were extracted from whole cells of E. coli using the phenol procedure of Zubay (1962). The sRNA was removed by molar sodium chloride precipitation of rRNA at 0° (Crestfield, Smith, and Allen, 1955). The

Figure 1



An elution profile illustrating the column chromatographic purification of *E. coli* sRNA by passage through DEAE-cellulose.

precipitate of rRNA was washed successively with 70% ethanol, 95% ethanol, and ether, and then air-dried. Precipitation of the rRNA at 0°, from aqueous molar sodium chloride solution, was performed three more times at an RNA concentration of 5 mg/ml. The final air-dried powder had an extinction coefficient (1%, 1 cm) of 220 when the absorbance was measured at 260 mμ in 0.05 M sodium chloride solution.

(2) Purification of E. coli sRNA Purchased From a Commercial Supplier.

E. coli B sRNA was purchased from General Biochemicals Ltd., and purified by column chromatography according to the procedure of Bell, Tomlinson, and Tener (1964). An aqueous solution containing about 900 mg of sRNA was applied to a 4.5 cm x 30 cm DEAE-cellulose column, and elution was begun with a linear gradient of 0.0 to 1.5 M sodium chloride in 0.0025 M Tris-chloride, pH 8.0. The total volume of the eluent was 8 liters. Sixty ml fractions were collected and the elution of sRNA from the column was monitored by following the absorbance of each fraction at 260 mμ. The elution profile obtained, is shown in Figure 1. The fractions 50-80, indicated in the diagram, were pooled, and the RNA was precipitated by the addition of 2.5 volumes of 95% ethanol. After storage at 4° for 48 hours, the RNA was sedimented by centrifugation and washed with cold

70% ethanol, 95% ethanol, and ether, and then air-dried. The column purified sRNA had an extinction coefficient (1%, 1 cm) of 222 when absorbance was measured at 260 m μ in 0.05 M sodium chloride solution. Sedimentation of a solution of E. coli sRNA in a Spinco Model E analytical ultracentrifuge, and observation with the schlieren optical system, revealed a single, relatively homogeneous boundary, with a calculated $S_{20,w}$ value of 4.1 (Figure 12c and d).

(iii) Methods

(1) Methods Employed in the Analysis of Alkali Hydrolyzates of RNA.

(a) Alkali Hydrolysis of RNA.

Four percent aqueous solution^s of ribonucleates were made 1 M with respect to alkali by the addition of 10 M sodium hydroxide solution. Hydrolysis was allowed to proceed at room temperature for 90 hours before the solution was neutralized with concentrated formic acid.

(b) Recovery of Alkali-Stable Dinucleotides by Anion-Exchange Fractionation of Alkali Hydrolyzates of RNA.

Neutralized hydrolyzates, containing the hydrolysis products from about 1 gram of RNA, were diluted with water to reduce the salt concentration below 0.025 M, and were then applied to 4.5 cm x 30 cm DEAE-cellulose columns (formate form). Stepwise elution of the hydro-

TABLE I

Eluents Used For Stepwise Elution of Nucleotides
and Dinucleotides From DEAE-cellulose

Compound	Net Charge at pH 7.8	Eluent
N	0	Water
Np	-2	0.085 <u>M</u> Tris-formate, pH 7.8, 7.3 <u>M</u> urea
NxpNp	-3	0.17 <u>M</u> Tris-formate, pH 7.8, 6.6 <u>M</u> urea
pNp	-4	1 <u>M</u> pyridinium for- mate, pH 4.5*

* This fraction would also include any alkali-stable tri-nucleotides present in the hydrolyzates.

lysis products from the anion-exchange column was accomplished by increasing the concentration of the competing anion in 6-8 M urea solutions (Tomlinson and Tener, 1963). The inclusion of urea reduces non-ionic interactions and allows the fractionation to proceed on the basis of the net charge of each of the components present in the hydrolyzate. Table I shows the eluents used to elute various types of compounds from DEAE-cellulose.

Alternatively, when large scale hydrolyzates were fractionated to obtain relatively large quantities of the dinucleotides, $N^4\text{MeCxpCp}$ and $N^6,N^6\text{diMeAp}N^6,N^6\text{diMeAp}$, a different procedure was followed. In this case, the nucleoside monophosphates were removed in the usual manner and the column was washed well with water, to remove all traces of salt and urea. Elution was then begun with 1 M formic acid and small fractions were collected and monitored at 260 $m\mu$ for the elution of $N^4\text{MeCxpCp}$, CxpCp , and $N^6,N^6\text{diMeAp}N^6,N^6\text{diMeAp}$. These fractions were then pooled, neutralized with pyridine, and evaporated to dryness. The salt-free residue was dissolved in water and the dinucleotides were resolved by paper chromatography (Singh and Lane, 1964). Each dinucleotide was eluted from the paper chromatogram, and desalted by charcoal adsorption and elution, according to the procedure described in section (iii) (1)(c), of this part of the thesis.

(c) Procedures Used to Desalt the Compounds Present in the Effluents From DEAE-cellulose Column Fractionations.

Nucleosides recovered in the initial effluent from DEAE-cellulose columns, were concentrated and desalted by adsorption to, and elution from, Norit A charcoal. Fifty mg charcoal discs were mounted in a 2.5 cm glass column between two layers of No. 545 celite. After adjusting the pH of the sample to 5.0, by the addition of acetic acid, the solution, containing up to 1 μ mole of nucleosides, was passed through the column. The column was then washed three times with 50 ml aliquots of water to remove residual salt, and the nucleosides were eluted with an eluent containing 90 ml of 50% ethanol and 10 ml of pyridine. The pyridine-ethanol effluent was evaporated in a flash evaporator at 40^o, and the dry residue was recovered in 200 μ l of water for paper chromatography.

Nucleoside diphosphates, eluted from the column with 1 M pyridinium formate, were recovered by removal of the volatile solvent in a flash evaporator. The resulting pyridinium salts were converted to ammonium salts by re-evaporation from 25 ml of 0.5 M ammonium hydroxide solution.

Mononucleotide and dinucleotide effluent fractions were desalted by diluting them five- and ten-fold, respectively, with water, and passing the diluted solutions

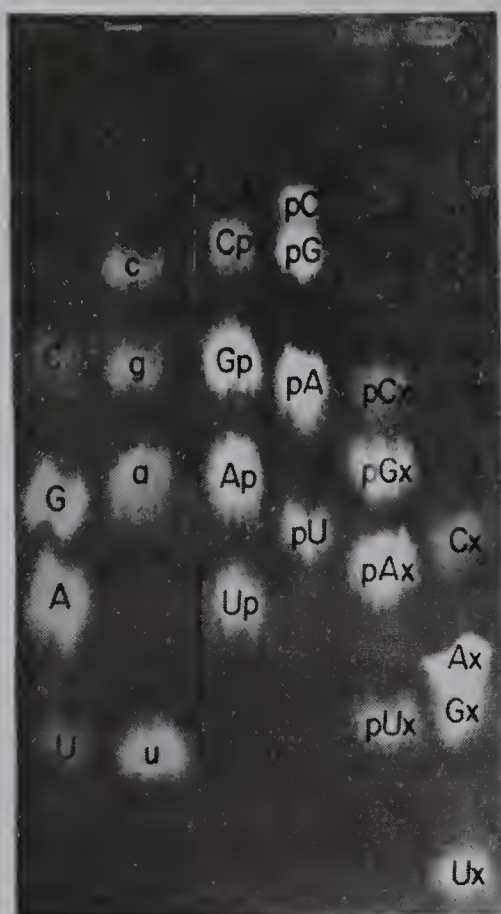
through another DEAE-cellulose (formate form) column. After washing the columns with large amounts of water, to remove residual urea and salt, the compounds were eluted with 1 M pyridinium formate, pH 4.5, and evaporated to dryness at 40° in a flash evaporator. The pyridinium salts of the compounds were converted to the ammonium salts in the manner employed for the nucleoside diphosphates (see above).

(d) Paper Chromatographic Resolution of Individual Dinucleoside Phosphates.

About 2 μ m of desalted dinucleotides were dissolved in 200 μ l of water, mixed with 20 μ l of 1 M ammonium formate buffer, pH 9.2 and 10 μ l of column purified E. coli phosphomonoesterase (Worthington Biochemicals Co.), and then incubated for 2.5 hours at 37°. The dinucleoside monophosphates of the general structure Nx_pN, which resulted from this treatment, were resolved by two-dimensional paper chromatography. The removal of the 2'(3')-phosphomonoester groups was previously found to result in an improved pattern of chromatographic resolution for these compounds (Singh and Lane, 1964).

The first-dimensional development was effected with a solvent composed of 80 volumes of 95% ethanol and 20 volumes of water, and was used with Whatman No. 1 paper, which had been impregnated with ammonium sul-

Figure 2



An ultraviolet contact photograph depicting the one-dimensional paper chromatographic separation of various compounds in system A. The origin is at the top of the photograph. (from Lane, 1965)

phate (Lane, 1963). The chromatography tank was pre-equilibrated with the vapor of the developing solvent. Figure 2 shows an ultraviolet contact photograph depicting the chromatographic separation of different nucleate derivatives in this system, which will be designated system A.

The second-dimensional development was effected with a solvent composed of 80 volumes of saturated ammonium sulphate solution and 2 volumes of isopropanol (see Markham and Smith, 1952). This will be designated paper chromatographic system B. In order to obtain a permanent record of each chromatogram, ultraviolet contact photographs were routinely made following chromatographic development, and drying of the chromatograms in air, at room temperature.

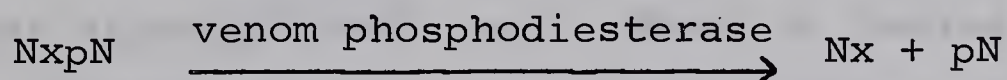
(e) Characterization of Alkali-Stable Dinucleotides.

The individual dinucleoside phosphates were located on paper chromatograms by their quenching of ultraviolet light from a hand-lamp. Appropriate areas of paper were excised, and eluted in water or in 0.1 M hydrochloric acid. Each of the compounds was characterized spectrally at pH values 2, 7, and 12 using a Bausch and Lomb Model 505 spectrophotometer between 220 m μ and 340 m μ . The recovered ultraviolet-absorbing material accounted for 85-90% of the material (260 m μ) originally

present in the dinucleotide fraction of alkali hydrolyzates. The quantity of each of the dinucleotides present, was calculated by employing a molar extinction coefficient, obtained by adding the molar extinction coefficients (260 mμ) of the constituent nucleosides of the corresponding normal dinucleotides, with no correction for hypochromicity.

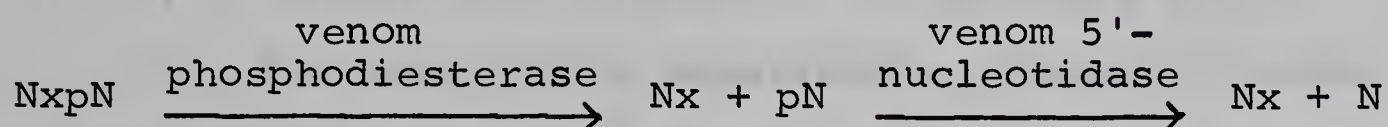
Each of the dinucleotides was further characterized by degradation with either purified snake venom phosphodiesterase or whole venom (Vipera russelli venom, obtained from Ross Allen's Reptile Institute, Inc.).

For phosphodiesterase hydrolysis, about 0.3 μmole of dinucleoside phosphate was dissolved in 200 μl of purified phosphodiesterase solution (50 μg of protein), and mixed with 20 μl of 1 M ammonium formate buffer, pH 9.2. Incubation was continued at 37° for 24 hours. The products of digestion, the normal 5'-nucleotide and 2'-O-methyl nucleoside, were formed in equimolar amount. Phosphodiesterase hydrolysis of a dinucleoside phosphate can be schematically illustrated as follows:



Alternatively, hydrolysis of a similar amount of dinucleoside phosphate was accomplished with whole snake venom to yield equimolar quantities of the consti-

tuent normal nucleoside and 2'-O-methyl nucleoside. Individual dinucleoside phosphates in 25 μ l of water were mixed with 25 μ l of 1 M ammonium formate buffer, pH 9.2, and 50 μ l of a 0.3% solution of viper venom. The digests were incubated at 37° for 24 hours. Whole venom digestion of a dinucleoside phosphate can be schematically illustrated as follows:



The products formed by hydrolysis with purified phosphodiesterase or whole venom, were resolved by one-dimensional paper chromatography in system A and characterized, both by their mobilities relative to marker compounds, and by their spectral characteristics at different pH values.

Individual dinucleoside phosphates could be further characterized by the electrophoretic properties of their hydrolysis products, in borate buffer (Smith and Dunn, 1959). Electrophoresis in 0.025 M sodium tetraborate buffer, pH 9.2, was performed in a Durrum-type paper electrophoresis cell, Model R, Series D, Beckman. In this system, normal and 2'-O-methyl nucleosides resolved into four well-separated bands which migrated in the following order of increasing mobility

toward the anode: (i) Ax, Cx, N⁴MeCx; (ii) Gx, Ux; (iii) A, C; (iv) G, U.

In addition, electrophoresis in 1 M formic acid, pH 2.0, was also useful for characterizing amino-substituted nucleosides. In this case, resolution into three well-separated bands was obtained. The following order of increasing mobility toward the cathode was obtained: (i) U, Ux; (ii) G, Gx; (iii) A, C, Ax, Cx, N⁴MeCx.

For deamination experiments, about 0.1 μ mole of nucleoside was dissolved in 700 μ l of water, and then mixed with 400 μ l of 5 M sodium nitrite and 100 μ l of glacial acetic acid. The reaction was allowed to continue overnight at room temperature. The pH value of the solution was adjusted to 5.0 before desalting on a charcoal column.

The 5,6-photohydrates of N⁴MeCx, Cx and C were formed by irradiating an aqueous solution of the nucleoside (0.1 μ mole per ml) in a quartz cuvette for 60 minutes, using ultraviolet light from a germicidal lamp, which emitted mainly 253.7 m μ wavelength.

(f) Methods Employed for Pseudouridylate Analyses.

For the analysis of pseudouridylate in rRNA, about 2000 μ moles of 2'(3')-mononucleotides, obtained from an alkali hydrolyzate of E. coli rRNA, was desalted

on a large DEAE-cellulose (formate form) column. The material, so obtained, was applied to a 2.5 cm x 20 cm column of Dowex-1. Chromatographic elution of Cp, A2p, and A3p was effected with 1.3 M acetic acid-0.01 M sodium acetate buffer, pH 2.8 (Cohen, 1954). Forty ml fractions were collected. Following the complete elution of A3p, the chromatographic elution of ψ p and Up was initiated by the passage of 1.0 M acetic acid-0.15 M sodium acetate, pH 3.5 into the column. After the complete removal of ψ p and Up, Gp was eluted from the column with 1.2 N hydrochloric acid. Pseudouridylate was eluted in a small peak immediately preceeding the large peak of Up. The nucleotide material in the small peak containing ψ p was freed of salt by charcoal adsorption, subjected to paper chromatography in system A, and the separated nucleotides were characterized spectrally at pH values of 2, 7, and 12.

Analysis for pseudouridylate in sRNA was accomplished by direct two-dimensional paper chromatography of about 8 μ moles of material (Hudson, Gray, and Lane, 1965) from a neutralized alkali hydrolyzate. The 2'- and 3'-isomers of pseudouridylate were well resolved from one another, and from the four major 2'(3')-mononucleotides. The amounts of pseudouridylate and other nucleotides present in the sample, were determined by

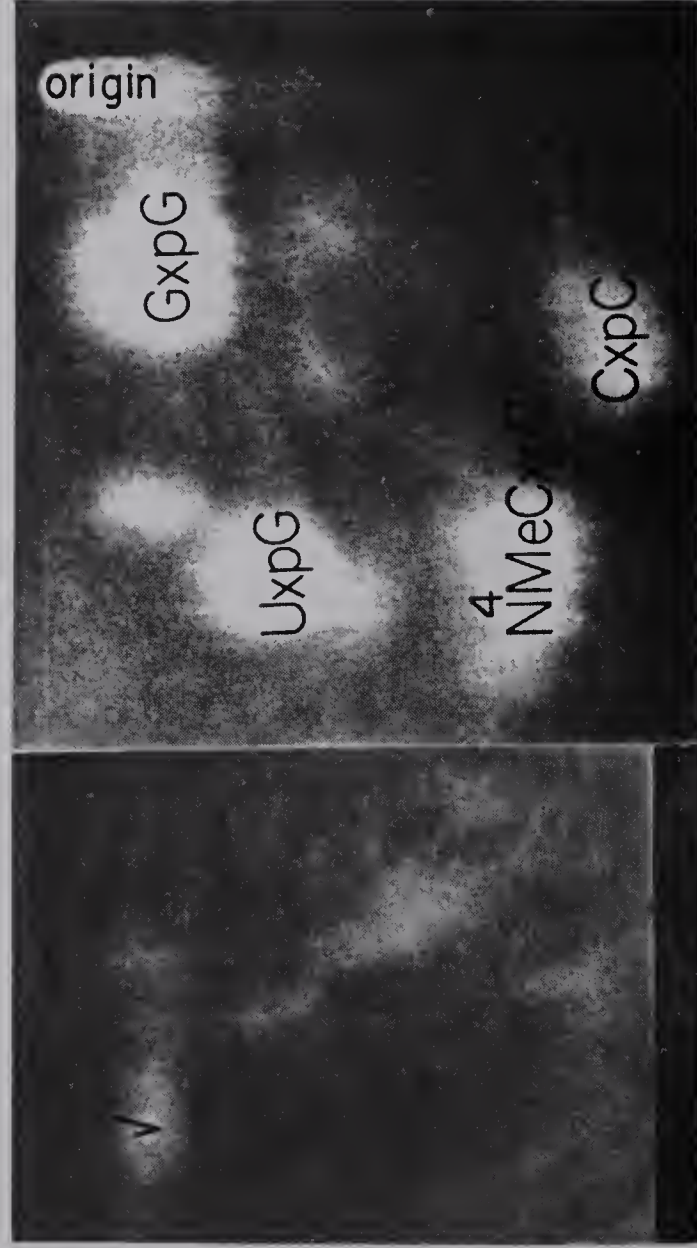
quantitative spectral analysis following elution in 0.1 M hydrochloric acid.

(2) Methods Employed in the Analysis of Snake Venom Hydrolyzates of RNA.

Two g of E. coli rRNA was dissolved in 200 ml of water, and then mixed with 100 ml of 1 M ammonium formate buffer, pH 9.2, and 100 ml of a 0.25% solution of Russell viper venom. The solution was incubated at 37° for 24 hours and then neutralized with formic acid. The neutralized hydrolyzate was fractionated into its nucleoside (N) and 2'-O-methyl nucleoside 5'-phosphate (pNx) components on a 4.5 cm x 15 cm DEAE-cellulose (formate form) column. The nucleosides were completely removed from the column with a water wash, and then the pNx compounds were eluted with 400 ml of 1 M pyridinium formate, pH 4.5. Pyridinium formate was removed by evaporation and the dry residue was re-evaporated from 25 ml of 0.5 M ammonium hydroxide solution.

The residue was dissolved in 100 ml of water and applied to a 2.5 cm x 10 cm Dowex-1 (formate form) column. Elution of pCx and pN⁴MeCx was begun with 0.5 M acetic acid. Small fractions were collected and monitored by absorbance between 220 and 340 mμ, until all material with a cytidine-like spectrum had been eluted. The pooled fractions were neutralized with pyridine and evaporated to dryness. The dry residue was dissolved

Figure 3a



Legend of Figure 3a

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of dinucleoside monophosphates derived from about 500 mg of E. coli rRNA. Two-dimensional chromatography was effected with system A in the first dimension (developed right to left in relation to the photograph), and with system B in the second-dimension (developed top to bottom in relation to the photograph). The compound in the area above UxpG was present in insufficient quantity to be identified. The dinucleoside phosphate designated "✓" in this photograph was identified as N⁶, N⁶diMeApN⁶, N⁶diMeA.

Figure 3b

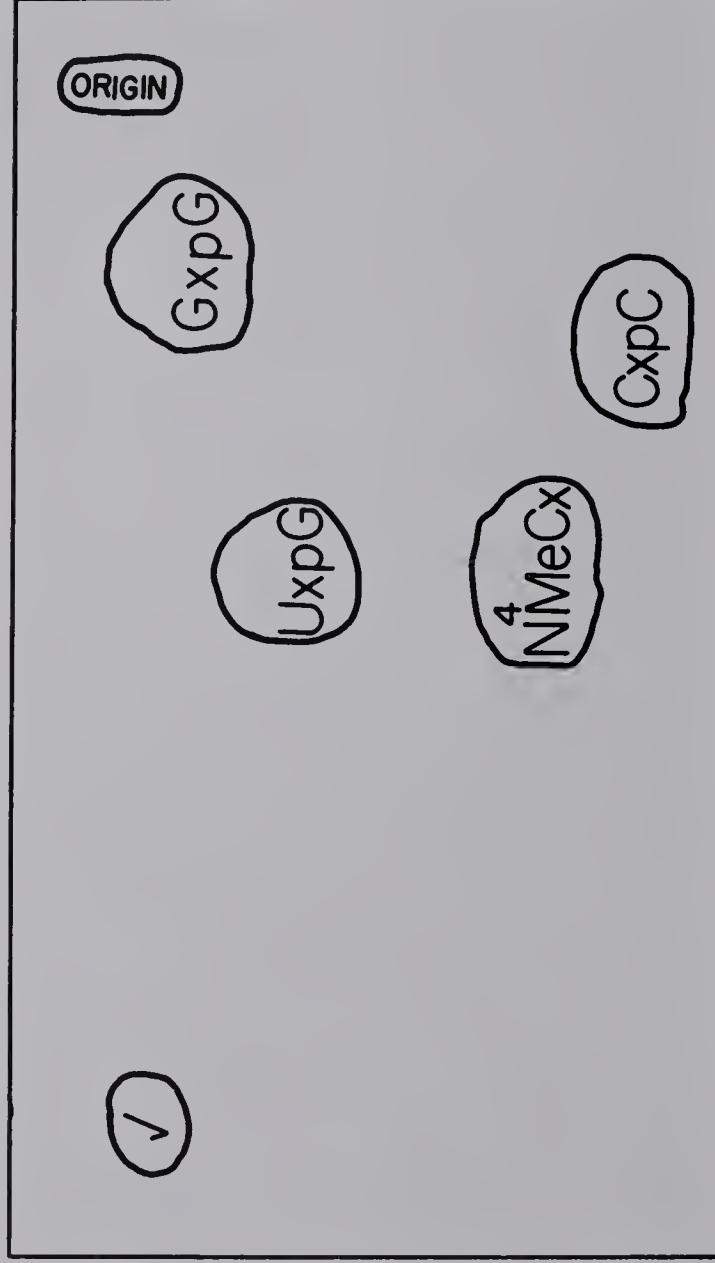
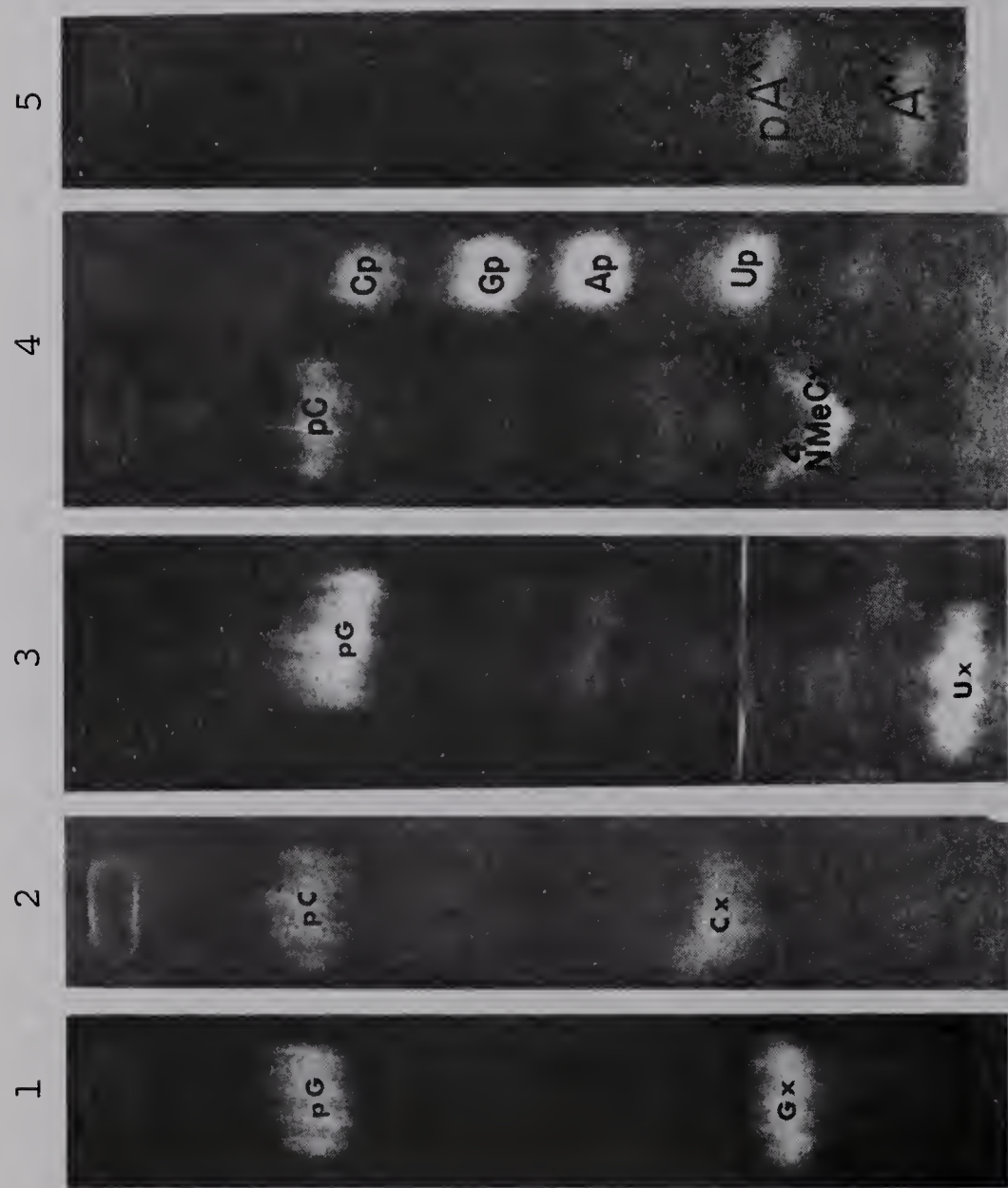


Diagram illustrating the two-dimensional paper chromatographic resolution of dinucleoside monophosphates from E. coli rRNA. The indicated areas were excised for analysis. See Figure 3a and legend.

Figure 4



Legend of Figure 4

Ultraviolet contact photographs depicting the one-dimensional paper chromatographic separations (system A) of the products formed by purified snake venom phosphodiesterase-hydrolysis, of each of the dinucleoside phosphates, isolated from E. coli rRNA. In each case, the origin is at the top of the photograph. The numbers 1, 2, 3, 4, and 5, at the top of the figure, indicate the hydrolyzates of GxpG, CxpC, UxpG, N⁴MeCxpC, and N⁶,N⁶diMeApN⁶,N⁶diMeA, respectively. The abbreviation pA^{xx} is used to represent the compound pN⁶,N⁶diMeA, and the abbreviation A^{xx} is used to represent the compound N⁶,N⁶diMeA. The unusual shape of the N⁴MeC_x spot is attributable to the fact that this hydrolyzate was not desalted prior to chromatography, and therefore contained a small amount of ammonium formate buffer, which interferes with migrations in system A. Marker compounds were chromatographed with each hydrolyzate, but to simplify the figure, these have been shown only for the chromatographic separation of the N⁴MeCxpC hydrolyzate. Since the digests were resolved on separate chromatograms, the mobilities in different digests are not rigorously comparable, owing to somewhat different development times used for the various chromatograms.

TABLE II

R_f Values for Various Compounds in

Type of Compound		R _f Values	
HETEROCYCLES	Cytosine	0.27	Guanine 0.38
NUCLEOSIDES	Cytidine C	0.37	Guanosine G 0.53
	2'-O-MeC	0.58	2'-O-MeG 0.71
	N ⁴ , 2'-O-diMeC	0.73	
NUCLEOSIDE-3'- PHOSPHOESTERS	Cp	0.24	Gp 0.37
	2'-O-MeCp	0.42	2'-O-MeGp 0.56
	2'-O-MeCpA	0.29	2'-O-MeGpA 0.34
	2'-O-MeCpG	0.19	2'-O-MeGpG 0.21
	2'-O-MeCpC	0.19	2'-O-MeGpC 0.20
	2'-O-MeCpU	0.42	2'-O-MeGpU 0.47
	N ⁴ , 2'-O-diMeCpC	0.40	
NUCLEOSIDE-5'- PHOSPHOESTERS	pC	0.20	pG 0.25
	TRISpC	0.06	TRISpG 0.08
	2'-O-MepC	0.40	2'-O-MepG 0.50
	N ⁴ , 2'-O-diMepC	0.66	1MepG 0.38
	5MepC	0.24	7MepG 0.10
			N ² -MepG 0.43
			N ² , N ² -diMepG 0.52
NUCLEOSIDE-3', 5'-DIPHOSPHO- ESTERS	pCp	0.10	pGp 0.14
	pCcp	0.19	pGcp 0.28
	ApCcp	0.18	
	GpCcp	0.11	
	CpCcp	0.09	
	UpCcp	0.22	
	2'-O-Me-ApCp	0.22	

Paper Chromatographic System A: 76% ethanol-water developing solvent used with Whatman #1 filter paper, impregnated with 0.6 M ammonium sulphate. The chromatograms were developed in a standard glass unit, pre-equilibrated with

Paper Chromatographic System A

R _f Values			
Adenine	0.50	Uracil	0.80
Adenosine A	0.64	Uridine U	0.80
2'-O-MeA	0.78	2'-O-MeU	0.96
N ⁶ ,N ⁶ -diMeA	0.87		
Ap	0.50	Up	0.64
2'-O-MeAp	0.64	2'-O-MeUp	0.76
2'-O-MeApA	0.46	2'-O-MeUpA	0.60
2'-O-MeApG	0.34	2'-O-MeUpG	0.47
2'-O-MeApC	0.30	2'-O-MeUpC	0.42
2'-O-MeApU	0.61	2'-O-MeUpU	0.78
N ⁶ -MeAp	0.80		
N ⁶ ,N ⁶ -diMeAp	0.86		
ApC	0.22		
pA	0.40	pU	0.57
TRISpA	0.16	TRISpU	0.24
2'-O-MepA	0.61	2'-O-MepU	0.78
N ⁶ -MepA	0.64	5MepU	0.63
1MepA	0.41		
N ⁶ ,N ⁶ -diMepA	0.72		
ApA	0.35		
pAp	0.23	pUp	0.37
ApAp	0.26	pUcp	0.52
2'-O-MeApAp	0.32		
pAcp	0.39		
ApAcp	0.28		
		Inosine pI	0.30
		Pseudouridine p _ψ	0.31
		p _ψ p	0.23

76% ethanol-water. The descending technique was employed, at room temperature, for 15-24 hours. A difference of 0.10 units in the R_f values is required for good resolution.

in 25 ml of 0.5 M ammonium hydroxide solution and re-evaporated to dryness. The dry residue was finally dissolved in 200 μ l of water and applied to filter paper for one-dimensional chromatographic separation in system A.

(iv) Results of Analyses

(1) Analyses of E. coli rRNA.

(a) Dinucleotides in Alkali Hydrolyzates of E. coli rRNA.

In order to obtain suitable quantities of dinucleotides for satisfactory spectral characterization, it was found necessary to hydrolyze 1.5 g of E. coli rRNA. Only four major alkali-stable dinucleoside phosphates could be detected on paper chromatograms following a two-dimensional separation. Figure 3a shows an ultraviolet contact photograph depicting the resolution of these four dinucleoside phosphates, and Figure 3b depicts the areas of the chromatogram which were excised.

Each of the dinucleoside phosphates GxpG, UxpG, N^4 MeCxpC, and CxpC was characterized by digestion with purified venom phosphodiesterase. Figure 4 shows the one-dimensional paper chromatographic separation of the hydrolysis products formed from each of the dinucleoside phosphates. In addition, the R_f values of each of the products of hydrolysis is presented in Table II, together with the R_f values of a variety of other nucleate deriva-

TABLE III

Dinucleotides and Pseudouridyate Recovered from
Large-scale Alkali Hydrolyzates of E. coli rRNA

Component*	Mole % of the Nucleotides	Mole % of the Dinucleotides
GxpGp	0.020	18
CxpCp	0.026	24
N ⁴ MeCxpCp	0.041	38
UxpGp	0.018	17
N ⁶ N ⁶ diMeApN ⁶ N ⁶ diMeAp	<u>0.003</u>	<u>3</u>
Total	0.11	100
Pseudouridyate	0.15	-

* The dinucleotide sequences listed were found to cumulatively account for more than 90% of the total ultraviolet-absorbance (260 mμ) in the dinucleotide fraction of the alkali hydrolyzates. The figures are the mean values obtained in three independent analyses of the dinucleotide fraction, recovered by alkali hydrolysis of 1.5 g samples of E. coli rRNA.

tives in chromatographic system A. In each case, the 2'-O-methyl nucleoside showed an enhanced chromatographic mobility with respect to the corresponding normal nucleoside in paper chromatographic system A (non-polar solvent). Each hydrolyzate contained only the 2'-O-methyl nucleoside and the normal 5'-nucleotide, and the compounds were present in equimolar proportions. Evidence for the identification of $N^4\text{MeCx}$ in these hydrolyzates is presented in section (iv)(d) of this part of the thesis.

The dinucleotides present in rRNA accounted for 0.11 mole % of the constituent nucleotides. The amounts of each of these compounds in rRNA are presented in Table III.

One additional dinucleotide, $N^6,N^6\text{diMeApN}^6,N^6\text{-diMeAp}$, was identified in this fraction, but it accounted for only 0.003 mole % of the constituent nucleotides. The dinucleoside phosphate, $N^6,N^6\text{diMeApN}^6,N^6\text{diMeA}$, had a very high R_f value in the non-polar-solvent system A used for first-dimensional development, but had a very low R_f value in the polar-solvent system B used for second-dimensional development. Unlike the other dinucleotides, which are completely stable in 1 M alkali, by virtue of the presence of a 2'-O-methyl substituent, this dinucleotide does not contain 2'-O-methyl ribose,

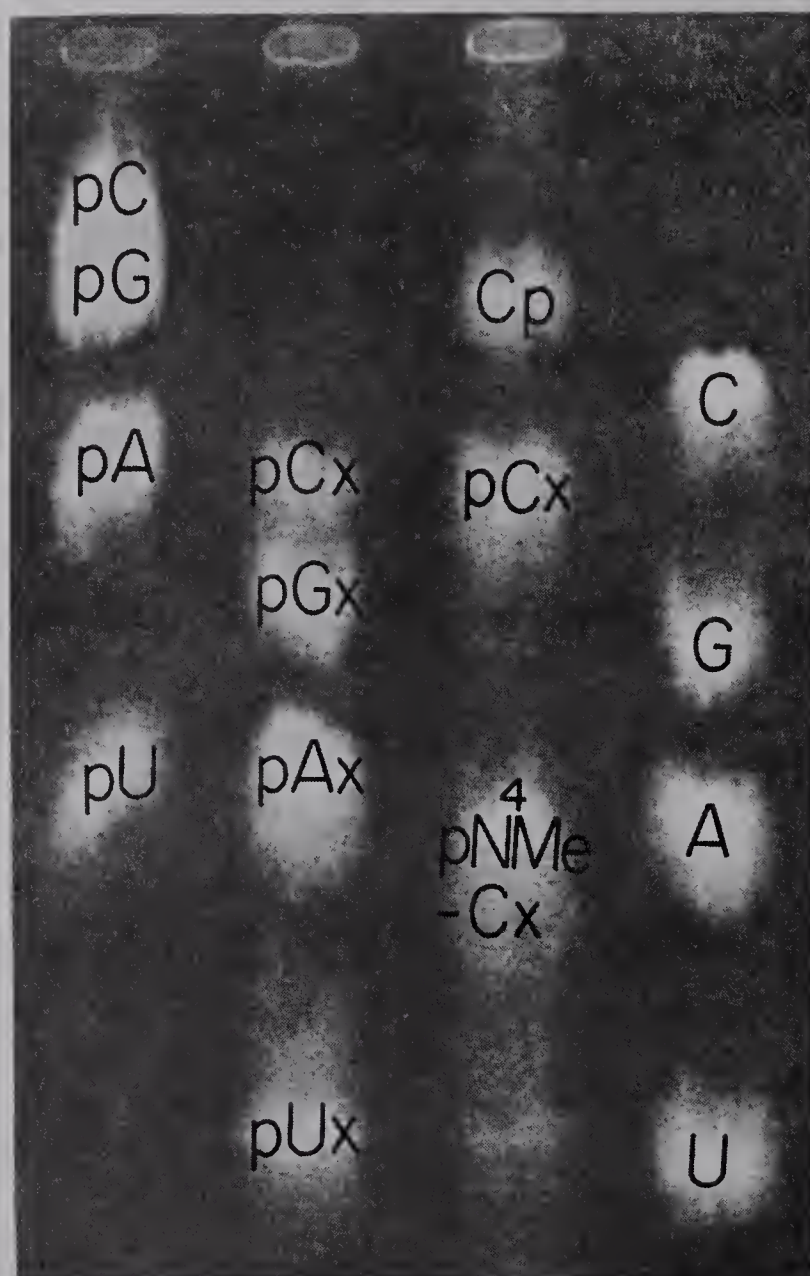
and it is present after 90 hours because of an extremely slow rate of hydrolysis. Treatment of the dinucleoside phosphate with purified snake venom phosphodiesterase, was found to produce equimolar amounts of a borate-complexing nucleoside and a nucleotide, which was converted to a borate-complexing nucleoside, by the 5'-nucleotidase in Russell viper venom.

The first-order hydrolysis constant for N^6, N^6 -diMeAp N^6, N^6 diMeA, in 1 M sodium hydroxide at room temperature, was determined to be $0.03-0.04 \text{ hr}^{-1}$.

The nucleoside and 5'-nucleotide produced by phosphodiesterase treatment of N^6, N^6 diMeAp N^6, N^6 diMeA, had spectra ($\lambda_{\text{max.}}$, pH 1.0 = 268 m μ ; $\lambda_{\text{max.}}$, pH 13.0 = 276 m μ) which were identical with those reported for N^6, N^6 -dimethyl adenosine (Littlefield and Dunn, 1958). Additional evidence for tetramethyl-substitution of this dinucleotide is presented in Part III of the thesis.

Because of the relatively slow rate of alkali hydrolysis for N^6, N^6 diMeAp N^6, N^6 diMeAp, it was possible to isolate this dinucleotide, as well as several other alkali-labile dinucleotides, from a 24 hr alkali hydrolyzate of E. coli rRNA. Incubation of the dinucleotides from the 24 hr hydrolyzate, for a further 90 hours in 1 M alkali, resulted in the complete hydrolysis of the alkali-labile dinucleotides, and the resulting mononuc-

Figure 5



An ultraviolet contact photograph depicting the chromatographic mobility of pN^4MeCx , relative to marker compounds, in system A. The origin of the chromatogram is at the top of the photograph. Cp is formed when RNA is digested by whole venom (Volkin and Cohn, 1954) and this accounts for its presence, together with pCx and pN^4MeCx , in whole venom digests of E. coli rRNA. It is formed by the action of a venom RNase present in Russell viper venom (McLennan, 1966).

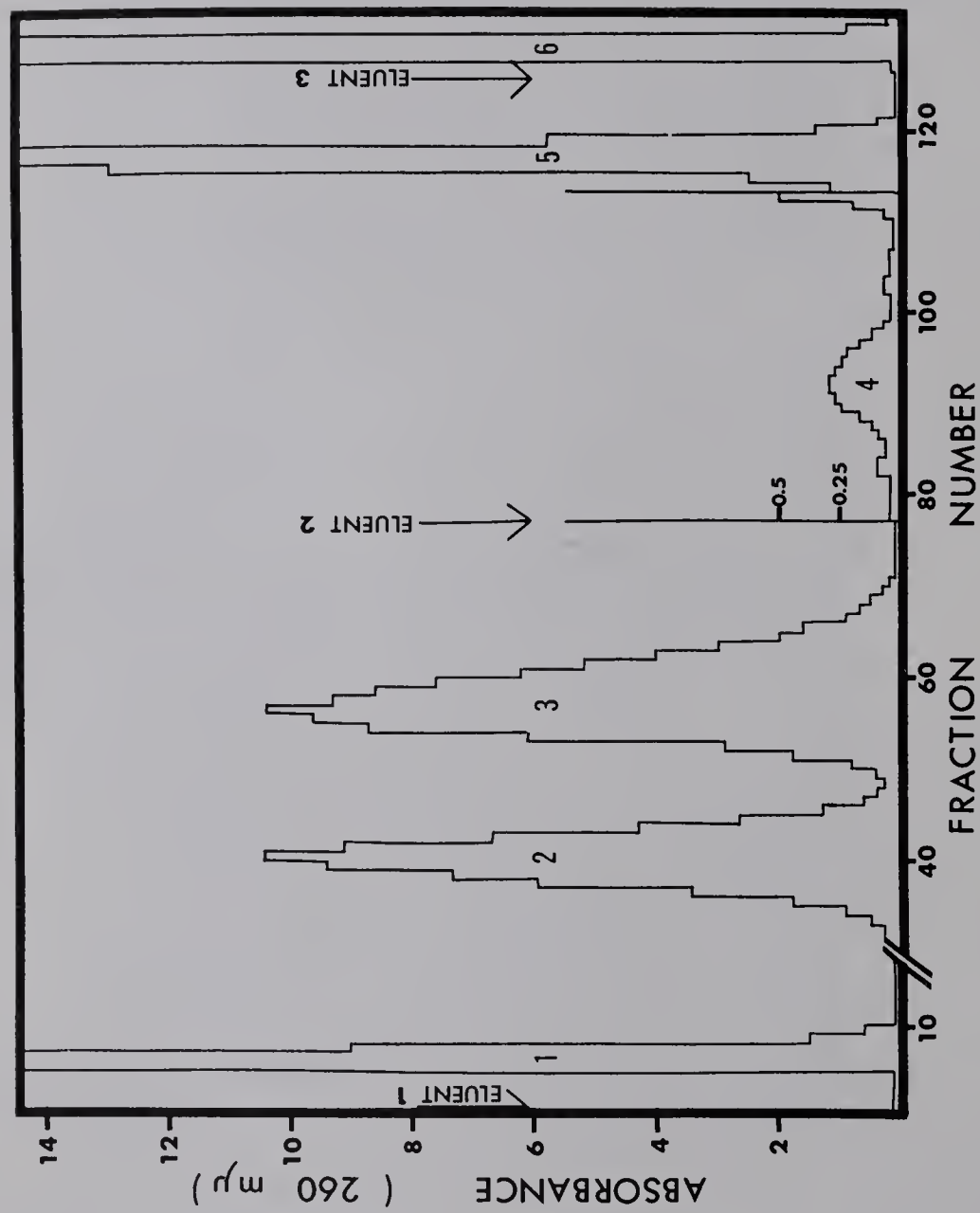
leotides were separated from alkali-stable dinucleotides, by fractionation on DEAE-cellulose. The ultraviolet contact photograph in Figure 8, shows the one-dimensional paper chromatographic resolution of N^6, N^6 diMeAp, and the four major mononucleotides obtained in this way. The large amount of Ap, relative to the other mononucleotides, can be attributed to the fact that ApNp-sequences, and in particular, ApAp and ApCp, are only very slowly hydrolyzed in alkali (Lane and Allen, 1961). For this reason, these alkali-labile dinucleotides constitute the bulk of the dinucleotides present in alkali hydrolyzates of E. coli rRNA, after 24 hr at room temperature in 1 M alkali.

(b) 2'-O-Methyl Nucleoside 5'-Phosphates in Snake Venom Hydrolyzates of E. coli rRNA.

The 2'-O-methyl nucleoside 5'-phosphates are present, together with the bulk of the normal nucleosides, in whole venom digests of RNA, because these O-methylated nucleotides are resistant to the action of the 5'-nucleotidase in snake venom. The pNx compounds are readily separated from the large bulk of normal nucleosides, by ion-exchange fractionation on DEAE-cellulose. This procedure has been used previously to isolate 2'-O-methyl nucleoside 5'-phosphates from wheat embryo sRNA (Hudson, Gray, and Lane, 1965).

Figure 5 shows the one-dimensional paper chromatographic mobility of pN^4 MeCx and pCx, isolated from

Figure 6



Legend of Figure 6

An elution profile illustrating the column chromatographic separation of pseudouridylate from the four major mononucleotides present in alkali hydrolyzates of E. coli rRNA. Descriptions of the Dowex-1 column, and the eluents employed, are presented in the text. In the inset, beginning with the addition of eluent 2, and ending just prior to the elution of peak 5, the absorbance-scale is expanded 4-fold, and the volume in effluent fractions is one-third as great as that in the part of the elution profile, not bounded by the inset. Peak 1, Cp; peak 2, A2p; peak 3, A3p; peak 4, ψ p; peak 5, Up; and peak 6, Gp.

whole venom digests of rRNA. The ultraviolet absorption spectra of pN^4MeCx , at pH values of 2, 7, and 12, were the same as those obtained for N^4MeCx , isolated by hydrolysis of the alkali-stable dinucleotide, $N^4MeCxpCp$. Electrophoresis in 1 M formic acid, pH 2.0 showed that pN^4MeCx had the same mobility as marker pC and pCx compounds. Following conversion of the 5'-nucleotide to a nucleoside, by treatment with E. coli phosphomonoesterase, the nucleoside migrated with the same mobility as C and Cx during electrophoresis in 1 M formic acid, as would be expected if the nucleoside were N^4MeCx . Further characterization of the 5'-nucleotide of N^4MeCx , as a (methyl- ^{14}C)-labeled component, is presented in Part III of the thesis.

(c) Pseudouridylate in Alkali Hydrolyzates of E. coli rRNA.

The anion-exchange column chromatographic separation of ψp from the remaining nucleotides in an alkali hydrolyzate of E. coli rRNA is illustrated by the elution diagram in Figure 6. Paper chromatographic resolution in system A, of nucleotide materials present in the leading, middle, and trailing regions of the ψp -peak showed, in addition to ψp , N^6MeAp , and an unidentified component with the spectral properties of Ap. The identification of N^6MeAp was based on the spectral data of Littlefield and Dunn (1958). About 0.017 mole

Figure 7

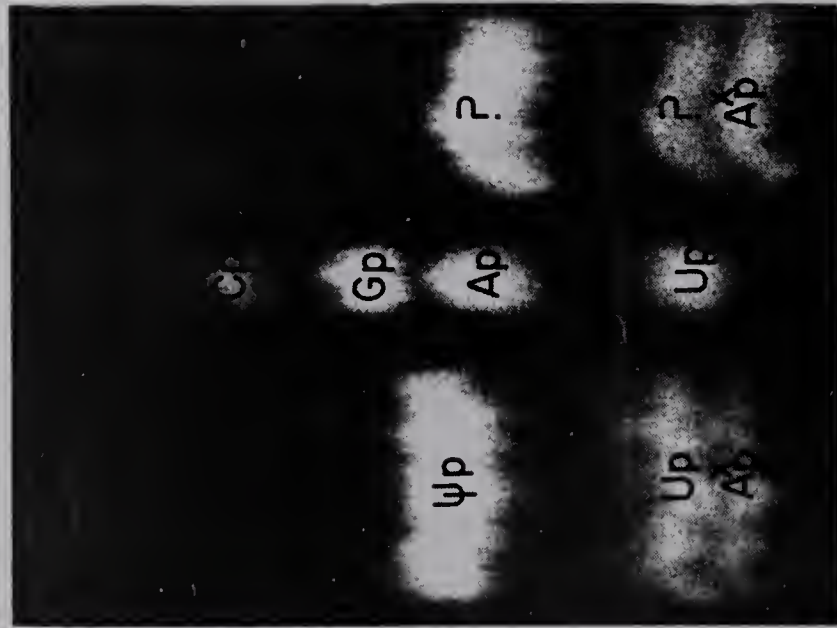
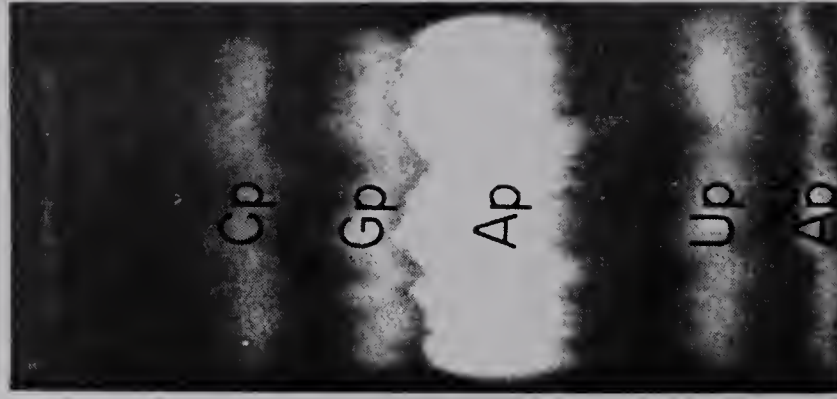


Figure 8



Legend of Figure 7

An ultraviolet contact photograph depicting the one-dimensional paper chromatographic separation (system A) of pseudouridylate from the remaining nucleotides present in the pseudouridylate peak eluted from a Dowex-1 column. Material from the middle of the pseudouridylate peak was chromatographed at the left of the marker mononucleotides. The origin is at the top of the photograph. Pseudouridylate, Up and N⁶MeAp(A^{Xp}) were identified as the three components present. Material from the leading edge of the pseudouridylate peak was chromatographed at the right of the marker mononucleotides and three ultraviolet-absorbing bands were observed. The band with the greatest mobility was identified as N⁶MeAp(A^{Xp}), but the remaining bands could not be identified. The band with the lowest R_f value (designated "?") had spectral properties similar to Ap. Material from the trailing region of the pseudouridylate peak was also chromatographed (not shown here), but it was found to contain only Up.

Legend of Figure 8

An ultraviolet contact photograph depicting the one-dimensional paper chromatographic separation (system A) of N⁶,N⁶diMeAp(A^{XXp}) from the four major mononucleotides. The origin is at the top of the photograph. The nucleotides were obtained by alkali hydrolysis of the dinucleotide fraction from a 24 hr alkali hydrolyzate of E. coli rRNA. The reason for the preponderance of Ap, relative to the remaining mononucleotides, is discussed in the text.

% of N^6 MeAp was found, but this may represent only a portion of the total amount of this compound in E. coli rRNA. The ψ p was spectrally pure judging by the spectra at pH values of 2, 7, and 12. It accounted for 0.15 mole % of the constituent nucleotides. Figure 7 shows an ultraviolet contact photograph of a paper chromatogram, in which material from the leading, and middle regions of the ψ p-peak were separated.

(d) Characterization of N^4 , 2'-O-Dimethyl Cytidine Derived From Alkali Hydrolyzates of E. coli rRNA.

Incubation of the dinucleoside phosphate, N^4 MeCxpC, in molar alkali for 90 hours at room temperature, failed to cause any hydrolysis. Thus, the presence of N^4 MeCxpCp in the dinucleotide fraction of alkali hydrolyzates of RNA, was not attributable to a slow rate of hydrolysis for a di-ribonucleotide, as in the case of N^6,N^6 diMeAp N^6,N^6 diMeAp, but was probably owing to the presence of a 2'-O-methyl substituent.

Evidence for 2'-O-methyl-substitution in the glycosyl of N^4 MeCx, also came from experiments employing borate electrophoresis. The products formed by venom phosphodiesterase-induced hydrolysis of N^4 MeCxpC, when subjected to electrophoresis in 0.025 M sodium tetraborate, pH 9.2, were resolved into one band with the spectral properties and mobility of pC, and a second band, which

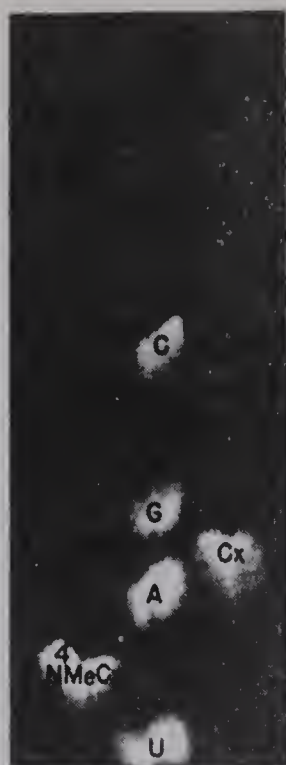
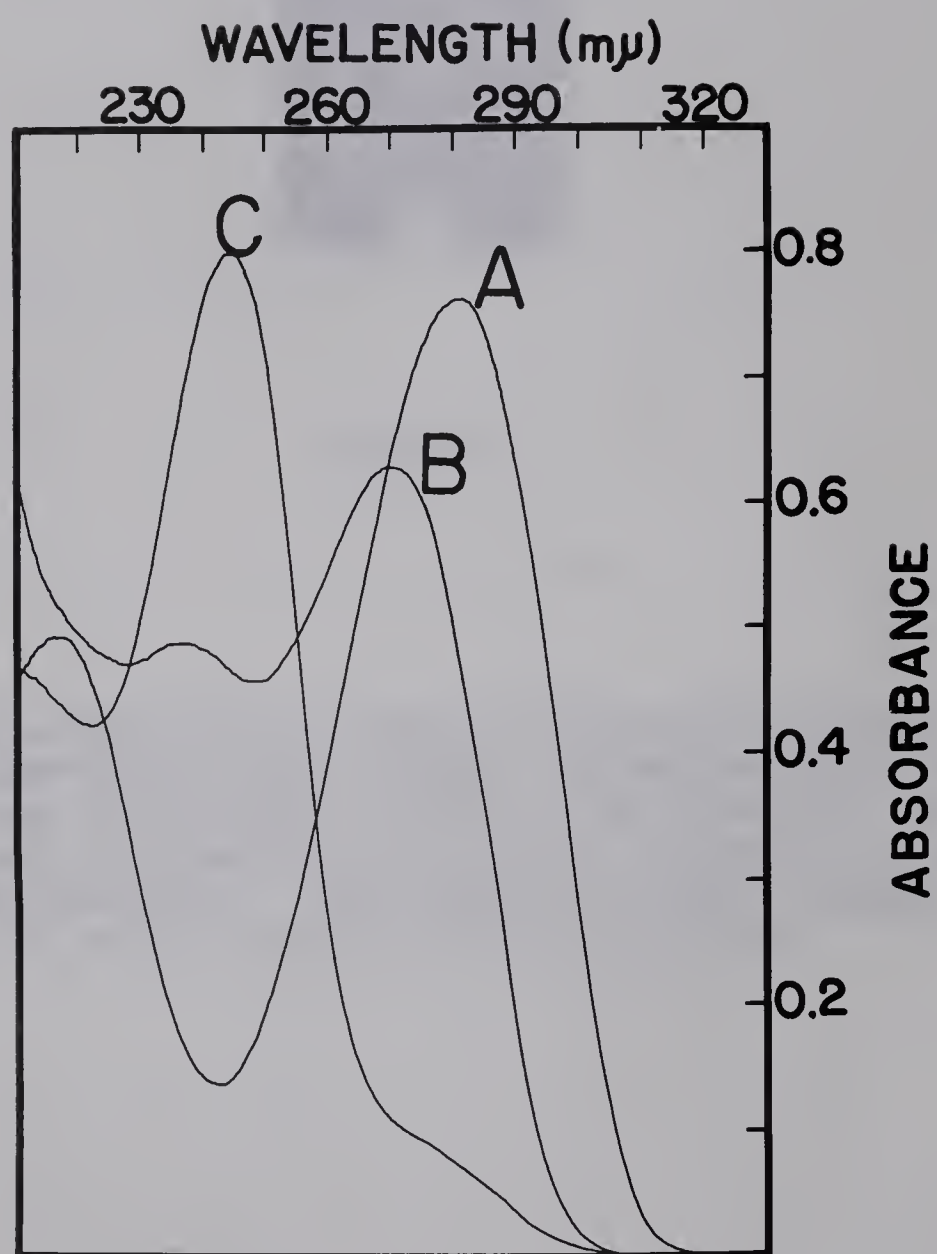


Figure 9

An ultraviolet contact photograph depicting the one-dimensional paper chromatographic mobilities (system A) of N^4 , 2'-O-dimethyl cytidine and 2'-O-methyl cytidine, relative to marker nucleosides. The origin of the chromatogram is at the top of the photograph.

Figure 10



Legend of Figure 10

Ultraviolet absorption spectra of $N^4,2'\text{-O-dimethyl}$ cytidine and its photolysis product. The curves are reproductions of the actual spectrophotometer-traces, obtained with a Bausch and Lomb 505 spectrophotometer. The spectrum of $N^4,2'\text{-O-dimethyl}$ cytidine is shown for pH 1.0 (A) and pH 7.3 (B), and the spectrum of the photolysis product is shown for pH 7.3 (C). A characteristic difference between the ultraviolet absorption spectra for cytidine and $N^4,2'\text{-O-dimethyl}$ cytidine is in the ratio $A(\text{max.}, \text{pH } 1.0)/A(\text{max.}, \text{pH } 7.3)$, which is 1.47 for cytidine, and 1.20 for $N^4,2'\text{-O-dimethyl}$ cytidine.

had the spectral properties of N^4MeC and the mobility of Cx. Similarly, whole venom digests of $N^4MeCxpC$, when subjected to electrophoresis in the same system, gave one band with the spectral properties and mobility of C, and a second band, which had the spectral properties of N^4MeC and the mobility of Cx. Thus the compound with the spectral properties of N^4MeC , had the same electrophoretic properties as Cx, and presumably possessed a 2'-O-methyl substituent (Smith and Dunn, 1959).

The presence of a methyl substituent in the heterocycle of N^4MeCx , was first indicated by the enhanced chromatographic mobility of N^4MeCx with respect to Cx in an organic solvent (Figure 9), and also by the enhanced mobility of $N^4MeCxpC$ with respect to CxpC in the first-dimension (non-polar solvent), and decreased mobility with respect to CxpC in the second-dimension (polar solvent) of two-dimensional paper chromatograms (Figures 3(a) and (b)).

Convincing evidence for the location of the heterocyclic methyl-substituent was obtained from ultraviolet absorbance measurements. The spectral characteristics of chromatographically purified N^4MeCx are shown in Figure 10, and they are in excellent agreement with the data of Szer and Shugar (1966), for the synthetically prepared N^4MeC . A detailed comparison of the spectral

TABLE IV

Spectral Comparison of N⁴,2'-O-Dimethyl Cytidine
and N⁴-Methyl Cytidine

Spectral Ratios (m _μ /m _μ)	N ⁴ MeC*	N ⁴ MeCx**	N ⁴ MeC*	N ⁴ MeCx**
	pH 1.0		pH 7.5	
230/260	0.83	0.79	0.88	0.87
240/260	0.37	0.37	0.91	0.88
250/260	0.49	0.49	0.84	0.83
270/260	1.70	1.65	1.15	1.15
280/260	2.11	1.99	0.91	0.92
290/260	1.71	1.66	0.37	0.34

* Estimated from the spectral data of Szer and Shugar (1966).

** Estimates based on chromatographically purified material from E. coli rRNA.

TABLE V

Spectral Characteristics of Some Methyl-Substituted Cytidine Derivatives

Compound	$\lambda_{\max.}(\text{acid})$	$\lambda_{\min.}(\text{acid})$	$\lambda_{\max.}(\text{neut.})$	$\lambda_{\min.}(\text{neut.})$	$\lambda_{\max.}(\text{alk.})$	$\lambda_{\min.}(\text{alk.})$
C ^a	280(212.5) ^b	241.5	271(229.5)	250.5(226)	273	251.5
C-5,6-hydrate ^c			240			
N ⁴ MeC ^d	280(216)	243	271(238)	250(228)	272(238)	250
N ⁴ MeC-5,6-hydrate ^c			245			
N ⁴ ,N ⁴ diMeC ^d	285(220)	245	278	240	280	245
N ⁴ ,N ⁴ diMeC-5,6-hydrate ^c			255			
Cx ^e	280	241.5	271	250.5	273	251.5
Cx-5,6-hydrate ^e			241			
N ⁴ MeCx ^e	280(216)	243	271(237)	250(228)	272(238)	250
N ⁴ MeCx-5,6-hydrate ^e			245			
3MeC ^d	277.5	245			265	247
5MeC ^f	288	245			278	255

^a Beaven, Holiday and Johnson in The Nucleic Acids, Vol. 1 (1955).^b Figures in parentheses indicate secondary maxima (or minima).^c Fikus, Wierzchowski, and Shugar (1962).^d Szer and Shugar (1966).^e Results of the present investigation.^f Fox et al. (1959).

properties of $N^4\text{MeCx}$ and $N^4\text{MeC}$ is presented in Table IV. Although $N^4\text{MeCx}$, Cx , and C exhibit similar spectra, $N^4\text{MeCx}$, unlike C and Cx , exhibits a well-defined secondary maximum (238 $m\mu$) at basic and neutral pH values. This secondary maximum is not present in the N^4, N^4 -dimethyl-substituted nucleoside (Szer and Shugar, 1966). $N^4, N^4\text{diMeC}$ has principal absorption maxima at 285 $m\mu$ (acid) and 278 $m\mu$ (neutral), which are significantly different from those exhibited by both $N^4\text{MeCx}$ and $N^4\text{MeC}$. Additional spectral characteristics presented in Table V serve to distinguish $N^4, 2'\text{-}\underline{\text{O}}$ -dimethyl cytidine from other methyl-substituted cytidine compounds.

Irradiation of aqueous solutions of pyrimidine compounds by ultraviolet light, results in the formation of a 5,6-hydrate through the addition of a water molecule across the 5,6-double bond of the heterocycle (Fikus, Wierzchowski, and Shugar, 1962; Moore and Thomson, 1955). The spectra of the photolysis products of C and Cx were found to have absorption maxima of 240 $m\mu$ and 241 $m\mu$, respectively, at neutral pH values, whereas the absorption maximum for $N^4, 2'\text{-}\underline{\text{O}}$ -dimethyl cytidine 5,6-hydrate was found to be 245 $m\mu$. This bathochromic and hyperchromic shift of the absorption spectrum for $N^4\text{MeCx}$ is illustrated in Figure 10. The data are in precise agreement with those of Fikus, Wierzchowski, and Shugar (1962) for the

5,6-photohydrate of synthetically prepared N^4 -methyl cytidine.

Substitution of the N^4 -position was confirmed by the observed resistance of N^4 MeCx, to deamination by nitrous acid. Cytidine and N^4 MeCx from whole venom digests of N^4 MeCxpc were resolved by paper chromatography, desalted on charcoal columns, and separately treated with nitrous acid. Each sample was desalted and subjected to electrophoresis in both 1 M formic acid, pH 2.0, and 0.025 M sodium tetraborate, pH 9.2. The compound remaining after nitrous acid treatment of N^4 MeCx, retained the electrophoretic and spectral properties of N^4 MeCx, whereas the compound remaining after nitrous acid treatment of C, exhibited the electrophoretic and spectral properties of uridine. One-dimensional paper chromatography of each of the nucleosides confirmed that C, but not N^4 MeCx had been deaminated. It is curious that spectral, chromatographic or electrophoretic evidence failed to indicate that N^4 MeCx had been nitrosylated, as might have been expected for the secondary amine. Presumably the nitroso-derivative was unstable, and decomposed to the parent amine, perhaps during electrophoresis in 1 M formic acid, pH 2.0.

Additional evidence for dimethyl substitution of $N^4,2'$ -O-dimethyl cytidine is presented in Parts II

TABLE VI

Alkali-stable Dinucleotides and Other Minor Components Recovered from Large-scale Alkali Hydrolyzates of E. coli sRNA

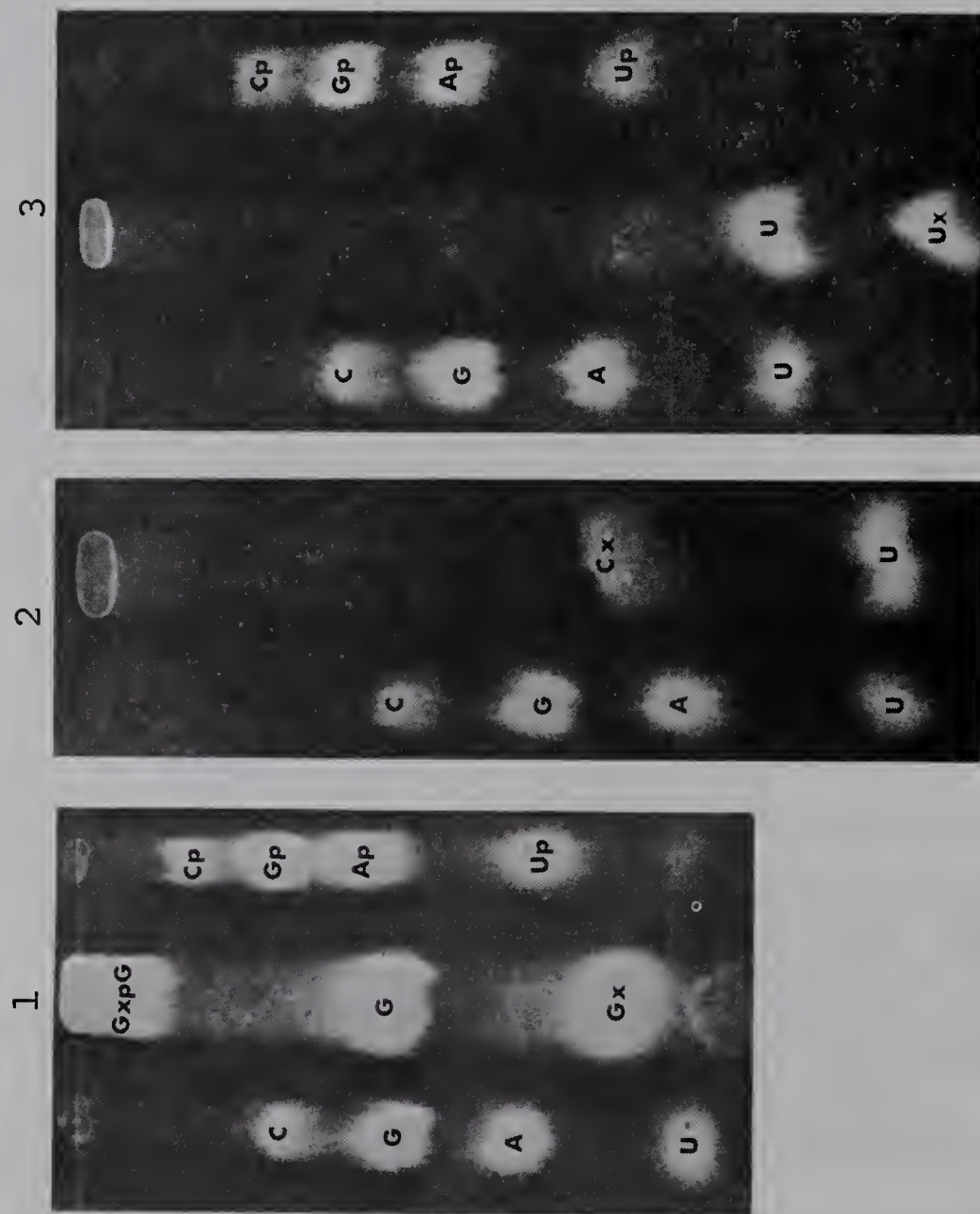
Component [*]	Mole % of the Nucleotides	Mole % of the Dinucleotides
<u>Dinucleotides</u>		
GxpGp	0.11	38
CxpUp	0.083	30
UxpUp	0.076	28
CxpAp-AxpCp	<u>0.011</u>	<u>4</u>
Total	0.28	100
<u>Mononucleotides</u>		
ψp	1.3	
Tp	1.2	
7MeGp ^{**}	<u>0.02</u>	
Total	2.5	
<u>Nucleosides</u>		
A	0.91	
G	0.02	
C	0.06	
U	<u>0.10</u>	
Total	1.09 ^{***}	

* The dinucleotide sequences listed were found to account, cumulatively, for more than 90% of the total ultraviolet absorbance (260 mμ) in the dinucleotide fraction of alkali hydrolyzates. The figures are the mean values for two independent analyses of the dinucleotide fraction, recovered by alkali hydrolysis of 600 mg samples of E. coli sRNA.

** Measured as an alkali-conversion product: 2-amino-6-hydroxy-5-methyl formamido-4-phosphoribosyl-aminopyrimidine. This probably accounts for only a small part of the total 7MeGp.

*** The chain length estimated from this value would be about $100/1.09 = \text{ca. } 91$ nucleotide residues, a degree of polymerization somewhat greater than found for sRNA specimens of animal and plant origin. However, the nucleotide composition of this E. coli sRNA preparation (19.1% Ap, 33.1% Gp, 26.8% Cp, 18.2% Up) was similar to that found for sRNA from animal and plant sources.

Figure 11



Legend of Figure 11

Ultraviolet contact photographs depicting the one-dimensional paper chromatographic separations (system A) of the products formed by snake venom hydrolysis of each of the dinucleoside phosphates, isolated from E. coli sRNA. In each case, the origin is at the top of the photograph. The numbers 1, 2, and 3 at the top of the figure, indicate the hydrolyzates of GxpG, CxpU, and UxpU, respectively. The hydrolysis of GxpG was incomplete after 24 hours, and, for this reason, GxpG appears on the chromatogram, together with the hydrolysis products.

and III of the thesis.

(2) Analyses of E. coli sRNA.

(a) Alkali-Stable Dinucleotides in Alkali Hydrolyzates of E. coli sRNA.

The alkali-stable dinucleotides accounted for about 0.3 mole % of the constituent nucleotides in E. coli sRNA (Table VI). Only one of these sequences, GxpGp, was also present in significant quantity in rRNA.

To characterize each of the dinucleoside phosphates, whole venom digestion was performed, followed by one-dimensional paper chromatography in system A, in order to resolve the two products. In each case, the two nucleosides derived from the dinucleoside phosphate, were found in equimolar proportions, and were identified by their chromatographic mobilities in system A, and by their spectral properties at different pH values. The ultraviolet photograph of Figure 11 shows the one-dimensional paper chromatographic separations of the products formed by digestion of each of the dinucleoside phosphates.

(b) Pseudouridylate and Other Minor Components in Alkali Hydrolyzates of E. coli sRNA.

The individual 2'- and 3'-phosphates of pseudouridine were well separated after two-dimensional paper chromatography of alkali hydrolyzates of E. coli sRNA, and they cumulatively comprised 1.3 mole % of the constituent nucleotides in E. coli sRNA. Ribothymidylate was also located on the same chromatograms, and was clearly

resolved from other nucleotide material. The ribothymidylate was identified on the basis of the spectral data of Littlefield and Dunn (1958), and comprised 1.2 mole % of the constituent nucleotides in E. coli sRNA.

Analysis of the nucleosides in alkali hydrolyzates of E. coli sRNA, by one-dimensional paper chromatography in system A, revealed four ultraviolet-absorbing bands. As expected for the alkali hydrolysis of sRNA, the predominant nucleoside was adenosine, derived from 5'-linked chain termini (Table VI). The adenosine, cytidine, and uridine bands were spectrally pure, but the "guanosine band" had an unusual spectrum. Following re-chromatography of the material in the "guanosine band", using the borate solvent of Plesner (1955), three bands appeared. These were identified as guanosine, 2-amino-6-hydroxy-5-methylformamido-4-phosphoribosylamino pyrimidine, and adenine. The pyrimidine compound is a breakdown product of 7-methylguanylate, produced by ring fission in alkali, and was identified by comparison with the spectral data of Lawley and Brookes (1963). The derivative of 7-methyl guanylate was found to account for 0.02 mole % of the total nucleotides in sRNA (Table VI). The presence of 7-methyl guanylate in E. coli sRNA has been reported previously (Dunn and Spahr, 1961). The breakdown product of 7-methyl guanylate has also been

TABLE VII

Quantitative Proportions for the Methyl-substituted Nucleotides and Pseudouridylation in rRNA and sRNA from Animal, Plant, and Microbial Cells

Unfractionated Specimen	RNA	Mole % of the Total Nucleotides Containing a			C-C glycosyl Bond (Pseudouridine)
		Methyl-substituted Heterocycle	Methyl-substituted Glycosyl	Methyl-substituted Heterocycle and Glycosyl	
Animal Cell (Liver)	sRNA	4.9 ^a	0.58 - 1.0 ^b	-	4.5 ^a
	rRNA	0.26 ^a	0.36 - 1.0 ^b	-	1.5 ^a
Plant Cell	sRNA	4.7 ^c	1.3 ^c	-	2.7 ^c
(Wheat Embryo)	rRNA	0.056 ^d	1.7 ^e	-	1.8 ^e
Bacterial Cell	sRNA	2.3 ^f	0.3 ^g	-	1.3 ^g
(<u>E. coli</u>)	rRNA	0.73 ^f	0.1 ^g	0.025 ^g	0.15 ^g
Yeast	sRNA	5.2 ^h	0.8 ^h	-	4.0 ^h
	rRNA	-	1.0 ^h	-	0.78 ⁱ

^a Values are for rat liver RNA, and methylated nucleosides included in the analysis were: N⁶MeA, N⁶,N⁶diMeA, 1MeG, N²MeG, N²,N²diMeG, and 5MeC (Dunn, 1959). The primary data of Dunn were presented relative to uridine, and have been re-calculated, relative to total nucleotides, using values of 18.2 mole % uridylation for rat liver sRNA (Osawa, 1960) and 19.4 mole % uridylation for rat liver rRNA (Hirsch, 1966).

^b The first value for sRNA is for sheep liver and the second value is for human liver. The first value for rRNA is for human liver and the second value is for sheep liver. Methylated nucleosides included in the analysis were: Ax, Gx, Cx, Ux, ψ x (Hall, 1964).

^c Methylated nucleosides included in the analysis were: 1MeA, N⁶MeA, 1MeG, 7MeG, N²MeG, N²,N²diMeG, 5MeC, 5MeU, Ax, Gx, Cx, Ux and ψ x (Hudson, Gray, and Lane, 1965).

^d The value is for 5MeC (C.A. Dekker, private communication).

TABLE VII, CONT'D.

- e Methylated nucleosides included in the analysis were: Ax, Gx, Cx, Ux and ψ x (Lane, 1965).
- f Methylated nucleosides included in the analysis were: 2MeA, N⁶MeA, N⁶,N⁶diMeA, 1MeG, 2MeG, 7MeG, 5MeU. 7MeG was estimated as an alkali-conversion product. rRNA is assumed to consist of 33%, 16S RNA + 67%, 23S RNA, on a weight basis (Dubin and Günalp, 1967).
- g Results of present study, and methylated nucleosides included in the analysis were: Ax, Gx, Cx, N⁴MeCx, and Ux.
- h Methylated nucleosides included in the analysis were: N⁶MeA, 1MeG, N²MeG, N²,N²di-MeG, 7MeG, 5MeC, 5MeU, Ax, Gx, Cx, Ux and ψ x (Gray and Lane, 1967).
- i Monier, Stephenson, and Zamecnik (1960).

found in alkali hydrolyzates of wheat embryo sRNA, in amounts similar to those detected in the present study (Hudson, 1965).

(v) Discussion

Stringent requirements are imposed on techniques designed to detect and characterize the extremely small amounts of 2'-O-methyl ribosyl constituents in E. coli rRNA. The occurrence of 2'-O-methyl ribosyl constituents as part of dinucleotides in alkali hydrolyzates, greatly facilitates their detection. In addition to the fact that dinucleotides can be easily separated from the bulk of the hydrolysis products (mononucleotides) by anion-exchange chromatography, it is noteworthy that the dinucleotides have an absorbance that is roughly twice as great as that for their 2'-O-methyl nucleoside constituents, and that this increased absorbance, greatly facilitates detection by ultraviolet quenching (Markham and Smith, 1950) on paper chromatograms. This is particularly important in the case of cytosine-containing compounds which can easily escape detection by ultraviolet quenching. The recovery of 2'-O-methyl ribosyl constituents as part of dinucleotides, also greatly expands the basis for characterization by allowing hydrolytic degradation to component 2'-O-methyl nucleoside and normal nucleoside or 5'-nucleotide.

As indicated by the data in Table VII, E. coli rRNA has a much lower proportion of 2'-O-methyl ribosyl

constituents, than do the rRNA specimens from animal and plant cells. As yet, it is not known whether this low 2'-O-methyl ribose content of E. coli rRNA, relative to animal and plant rRNA, is a general characteristic of rRNA from bacterial cells.

There are 16 possible alkali-stable dinucleotide sequences of the type NxpNp, in which N is any of the four major ribonucleoside constituents of RNA, and Nx is the 2'-O-methyl derivative of any of the four principal ribonucleosides. All of these 16 sequences are found in rRNA, and more than 10 of them are found in sRNA, from both wheat embryo and yeast. By contrast, there are only three of these sequences, in significant quantity, in the sRNA (GxpGp, CxpUp, and UxpUp) and rRNA (GxpGp, CxpCp, and UxpGp) from E. coli. Most notably, the sequence N⁴MeCxpCp, which is found in E. coli rRNA, has not been found in the rRNA from animal or plant cells.

The component N⁴MeCx, constitutes the first example of a compound in which there is methyl-substitution of both heterocycle and glycosyl of the same nucleoside. Neither the dinucleotide (N⁴MeCxpCp) nor the dimethyl-substituted nucleoside (N⁴MeCx) has been encountered elsewhere as a natural product, or as a product of organic synthesis. The identification was facilitated by the fact that there are only four major dinucleotide

sequences in E. coli rRNA, and thus the $N^4\text{MeCxpCp}$ dinucleotide did not escape detection by being "masked" by large amounts of other dinucleotides. The isolation of $N^4\text{MeCxpCp}$ from alkali hydrolyzates, together with the isolation of $pN^4\text{MeCx}$ from whole venom digests, provides evidence that the nucleoside is a bona fide component of E. coli rRNA, and is involved in the customary internucleoside 3'-5'-phosphodiester linkage in the ribonucleate chains. Since $N^6\text{MeAp}$ can arise from $l\text{MeAp}$ in alkali (Brookes and Lawley, 1960; Dunn, 1961), it might be conjectured that $N^4,2'\text{-O-dimethyl cytidine}$ could arise from $3,2'\text{-O-dimethyl cytidine}$ during exposure to alkali. The recovery of $N^4,2'\text{-O-dimethyl cytidine 5'-phosphate}$ from enzymic digests does not rule out such a possibility, since it has been found that at the pH value of 9.2, used for hydrolysis with snake venom enzymes, there is an extensive conversion of $p\text{lMeA}$ to $pN^6\text{MeA}$ (Hudson, Gray, and Lane, 1965). However, the alkali-catalyzed deamination of 3MeC to 3MeU (Hall, 1963) would tend to indicate that $N^4,2'\text{-O-dimethyl cytidine}$ does not arise from $3,2'\text{-O-dimethyl cytidine}$ by an alkali-catalyzed re-arrangement.

The available analytical data for sRNA and rRNA from yeast and wheat embryo, show that the relative proportions of the $2'\text{-O-methyl nucleosides}$ bear

no obvious relation to the proportions of the corresponding normal nucleoside constituents in the RNA specimens. The present study has shown that E. coli sRNA and rRNA are no exception in this regard, since 2'-O-methyl adenosine is virtually absent from both. The 2'-O-methylation of RNA is therefore a highly specific process, which results in a characteristic pattern of alkali-stable dinucleotide sequences in the sRNA and rRNA from different cells.

The quantity of ribothymidylate (1.2 mole %) in the preparation of E. coli sRNA, examined in this study, is in excellent agreement with the values reported for E. coli sRNA by Dunn, Smith, and Spahr (1960) and Dubin and Gunalp (1967). Similarly, the pseudouridylate content found for E. coli sRNA (1.3 mole %) is in good agreement with the value of Dubin and Gunalp (1967), but somewhat lower than the value obtained by Dunn et al. (1960) for E. coli sRNA. The amount of pseudouridylate found in E. coli rRNA is close to that reported for the analysis of 70S ribosomes (Dunn et al., 1960).

It has been noted earlier, in the case of 18S + 28S ribonucleates from wheat embryo, that the relatively high proportions of pseudouridine (1.8 mole %) and 2'-O-methyl ribose (1.7 mole %) could be

indicative of a structural, and/or biosynthetic, relation between them (Lane, 1965; Hudson et al., 1965). The analytical correlation noted with rRNA from wheat embryo can now be extended to the rRNA of E. coli, where a much lower content of pseudouridine (0.15 mole %) is associated with a correspondingly lower proportion of 2'-O-methyl ribose (0.11 mole %).

The isolation of N^6, N^6 diMeAp from E. coli rRNA has been reported by many different workers (Starr and Fefferman, 1964; Dubin and Günalp, 1967). The isolation of this component from E. coli rRNA, as part of an homologous dinucleotide, has not previously been reported. The first order hydrolysis rate for N^6, N^6 diMeAp N^6, N^6 diMeA ($k = 0.03-0.04 \text{ hr}^{-1}$), can be used in conjunction with the measured amount of the dinucleotide in a 90 hour hydrolyzate of RNA, to estimate a "zero-time" value for the amount of N^6, N^6 diMeAp in the RNA. On this basis, the amount of N^6, N^6 diMeAp in the E. coli rRNA specimen examined in this study, was 0.08 mole % of the constituent nucleotides. Dubin and Günalp (1967) have found that N^6, N^6 diMeAp is present only in the 16S component of E. coli rRNA. Since the preparation of E. coli rRNA examined here, contained about 50-60% 16S RNA and 50-40% 23S RNA, then, from the present findings, the amount of N^6, N^6 diMeAp in 16S RNA would be about $10/6 (10/5) \times 0.08$.

= 0.13 (0.16) mole %. This value corresponds well with Dubin and Gunalp's figure of 0.14 mole %, for the amount of N^6,N^6 diMeAp in E. coli 16S RNA, and it is concluded that all of the N^6,N^6 diMeAp in 16S RNA is probably present in the form of an homologous dinucleotide sequence, since 0.14 mole % corresponds to about 2 moles of N^6,N^6 -diMeAp per mole of 16S RNA. This sequence would be a strong hydrophobic center in a ribonucleate chain and its inability to participate in hydrogen bonding would seem to make it particularly interesting in regard to recent views concerning the role of base-methylation in abolishing messenger function in rRNA (Nakada, 1965).

The compound referred to on page 34 as the nucleotide conversion product is probably the free base which results from N-glycosyl cleavage of 7MeGp in alkali (D.B. Dunn, personal communication).

Part II. The Alkali-stable Dinucleotide Sequences in Each of the 16S and 23S Components of Escherichia coli RNA.

(i) Introduction

Techniques currently available for separating the 16S and 23S components of rRNA are limited to relatively small amounts of material. This is an important limitation for studies which are designed to measure the trace components found in each of the 16S and 23S RNA components. Thus, it was not feasible to prepare the individual 16S and 23S components of E. coli rRNA, in gram-quantities, and to examine each of them for alkali-stable dinucleotides, using the spectral techniques applied to gram-quantities of the unresolved rRNA components in the studies described in Part I of this thesis. For this reason, uniformly labeled ^{14}C -rRNA was prepared in milligram-quantities, and resolved into 16S and 23S components, by sucrose density-gradient centrifugation. The alkali-stable dinucleotide sequences, containing 2'-O-methyl ribose, were then characterized with the aid of carrier dinucleotides added to alkali hydrolyzates of the individual ^{14}C -labeled 16S and 23S RNA components.

Since RNA accounts for about 2.5%, and DNA accounts for about 0.5% of the wet weight of E. coli, it is possible to recover about 9 mg of nucleates, containing 7.5 mg RNA (6 mg rRNA), from 300 mg of E. coli. By

equally distributing the 9 mg of nucleates between two 27 ml centrifuge tubes, in the large SW-25 rotor of the Beckman Model L centrifuge, it is possible, by a single sucrose density-gradient centrifugation, to obtain satisfactory resolution of the mixed nucleates (DNA, sRNA, and 16S and 23S rRNA). Accounting for preparative losses incurred during purification and precipitation steps, following the initial sucrose density-gradient centrifugation, at least 1 mg of each of the purified 16S and 23S rRNA components can be recovered from 300 mg E. coli. For reliable analyses of the principal alkali-stable dinucleotide sequences, and chain termini (see Part V), a measurable radioactivity of about 500 cpm per compound is satisfactory, and since each of the principal alkali-stable dinucleotide sequences and chain termini accounts for roughly 0.025 mole % of the component nucleotides in E. coli rRNA, then the sample from which the dinucleotides are recovered, should have a measurable radioactivity of about $500 \times 100/0.025 = 2 \times 10^6$ cpm, or about 1 μ curie. Thus, it was necessary to devise conditions whereby each of the purified 1 mg samples of 16S RNA and 23S RNA, recovered from 300 mg of E. coli, would have a specific activity of 1 μ curie/mg.

A specific activity of 1 μ curie/mg of uniformly labeled ^{14}C -RNA corresponds to 1 μ curie/ca. 3 μ moles of

constituent nucleotides in the RNA, or about 1 μ curie/27-30 carbon/ ^{μ} atoms in the RNA. Thus, expressed in terms of the carbon atom, the requisite specific activity for the uniformly labeled ^{14}C -RNA preparations would be about 0.03 μ curie/carbon/ ^{μ} atom.

It was decided that, in order to make efficient use of the ^{14}C -precursor, and to achieve the requisite specific activity for the rRNA, a 300 ml culture, containing a small inoculum should be grown on a minimal medium, containing uniformly labeled ^{14}C -glucose, to a concentration of 1 mg of cells per ml. From such a culture it would be possible to obtain 300 mg of cells by supplementing the medium with 1 mg of glucose per ml of culture medium (Roberts et al., 1957). In this way the specific activity of the carbon-source would be maximal. By supplementing 300 ml of minimal medium with 300 mg of non-radioactive glucose and 0.5 mg of uniformly labeled ^{14}C -glucose, containing 0.5 mcurie, the carbon source for synthesis of cellular components would have a specific activity of 500 μ curie/300.5 mg glucose (= 500 μ curie/1669 μ moles glucose = 500 μ curie/10014 μ moles carbon), or 0.05 μ curie/carbon/ ^{μ} atom. Consequently, the 300 mg of E. coli could be expected to have a specific activity of about 0.05 μ curie/carbon/ ^{μ} atom in the cellular constituents. For a counting efficiency of about 60%, the

measurable radioactivity of the isolated rRNA would be $0.60 \times 0.05 = 0.03 \mu\text{curie/carbon}^{\mu}\text{atom}$, and this would satisfy the requirement, mentioned earlier, for satisfactory analyses of the principal component alkali-stable dinucleotide sequences, and chain termini, in 1 mg of uniformly labeled ^{14}C -rRNA from E. coli.

With due allowance for non-quantitative recoveries, this experimental plan was adopted, and proved to be successful, for the experiments to be described in this part of the thesis, and also, for the experiments to be described in Part V of the thesis.

(ii) Methods

(1) Organism and Growth Conditions.

E. coli B was maintained on Trypticase Soy Broth (Baltimore Biological Laboratory) by serial transfer at two week intervals. When cultures of the organism were required for the isolation of RNA, the minimal medium C of Roberts et al. (1957) was used with glucose as an energy source. This medium contained, in one liter, the following ingredients:
 NH_4Cl , 2g; Na_2HPO_4 , 6g; KH_2PO_4 , 3g; NaCl , 3g; MgCl_2 , 0.01g; Na_2SO_4 , 0.026g. To eliminate the lag phase during culturing, the inoculum for such cultures was obtained from an 8 hour culture grown on the medium C

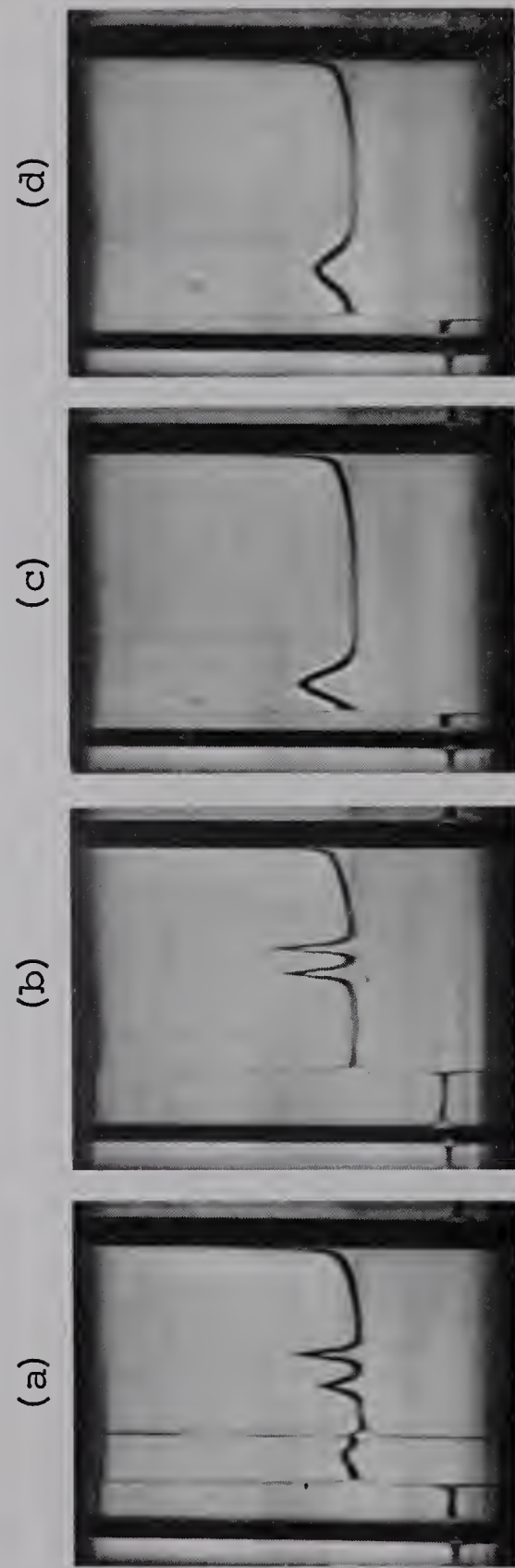
supplemented with 10 mg glucose per ml.

For the isolation of uniformly labeled ^{14}C -rRNA, a loop inoculum of cells was added to 300 ml of medium C, which had been supplemented with 300 mg of non-radioactive glucose and 0.5 mc (0.5 mg) of uniformly labeled D-glucose- ^{14}C (purchased from New England Nuclear Corp.). The organism was grown under forced aeration at 37° , and three 10 M sodium hydroxide traps were used to retain $^{14}\text{CO}_2$. Growth was followed spectrophotometrically at 600 $\text{m}\mu$ until exponential growth stopped (Optical Density at 600 $\text{m}\mu$ = 0.35 to 0.40; $4-5 \times 10^8$ cells/ml). The cells were then immediately poured over crushed ice. The cell suspension was centrifuged at 0° and 10,000 x g for 20 minutes, and the pellet was suspended in cold 0.01 M Tris chloride-0.05 M potassium chloride, pH 7.4. The cell suspension was recentrifuged and the resulting pellet was stored at -20° .

(2) Isolation of Uniformly Labeled ^{14}C -nucleates From E. coli.

About 400 mg of frozen cells were disrupted by grinding at 4° for 20 minutes with 1 g of alumina which had been previously washed with water (Allende, Monro, and Lipmann, 1964). The broken cells and alumina were suspended in 4 ml of 0.01 M Tris chloride-0.01 M magnesium chloride, pH 7.4, and the suspension was mixed with an equal volume of water-saturated phenol. The phenol

Figure 12



Legend of Figure 12

Schlieren photographs depicting the sedimentation of E. coli nucleates in a Spinco Model E analytical ultracentrifuge. Sedimentation was performed in 0.15 M sodium chloride solution with a ribonuclease concentration of ca. 0.25%. The bar angle was 45°.

(a) Photograph depicting the sedimentation of E. coli nucleates obtained by alumina-grinding and phenol extraction of cells. Photograph was taken 16 minutes after reaching a speed of 59,780 r.p.m.

(b) Photograph depicting the sedimentation of E. coli rRNA, following its precipitation from sodium chloride solution, to remove sRNA and DNA. Photograph was taken 16 minutes after reaching a speed of 59,780 r.p.m.

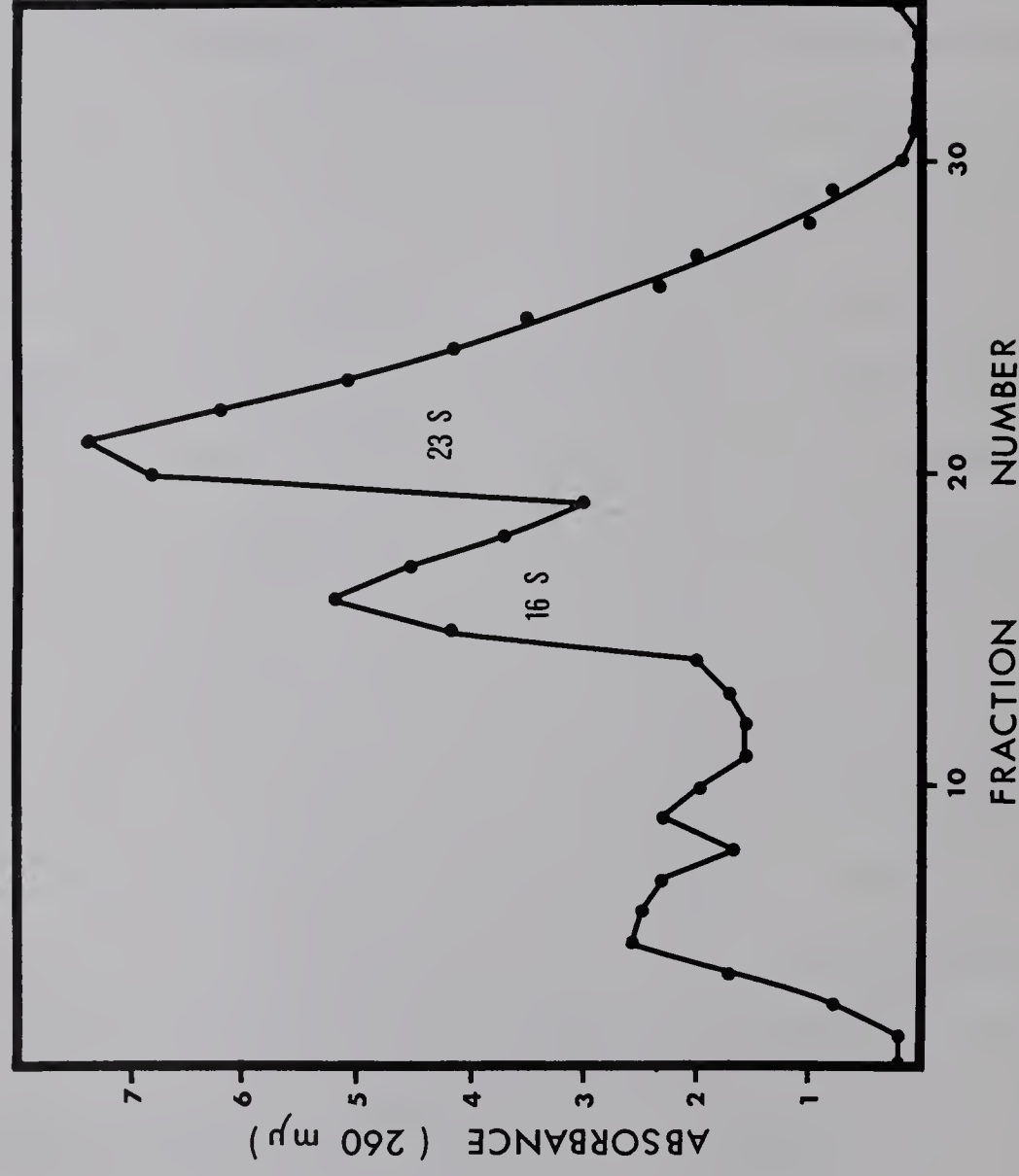
(c) and (d) Photographs depicting the sedimentation of E. coli sRNA after DEAE-cellulose column purification. The photographs of Figures(c) and (d) were taken 16 and 32 minutes, respectively, after reaching a speed of 59,780 r.p.m.

emulsion was shaken for 20 minutes at room temperature on a horizontal shaker and was then centrifuged at 2,000 x g. The upper aqueous layer was extracted three more times in the same way with water-saturated phenol, and then mixed with 2.5 volumes of cold 95% ethanol to precipitate the nucleates. The precipitate was sedimented by centrifugation at 0° and 12,000 x g for 20 minutes, and the pellet was washed three times in succession with 70% ethanol, 95% ethanol, and ether. The powder was air-dried and stored at -20°. At this stage, examination of the nucleates in the analytical ultracentrifuge revealed the presence of sRNA, DNA, and the 16S and 23S ribosomal RNA components (Figure 12a). The sRNA and DNA components could be effectively removed by precipitation of the rRNA, at 0°, from aqueous 2 M sodium chloride solution, in which sRNA and DNA are soluble. Examination of the rRNA components in the analytical ultracentrifuge, following precipitation from sodium chloride solution, illustrated the effective removal of sRNA and DNA (Figure 12b).

(3) Separation and Purification of the Uniformly ¹⁴C-labeled 16S and 23S rRNA Components of E. coli.

Preliminary experiments with the RNA isolated from unlabeled cells, showed that sucrose density-gradient resolution of the ribosomal ribonucleates into 16S and 23S components was much better if the rRNA had not previously been precipitated from 2 M sodium chloride

Figure 13

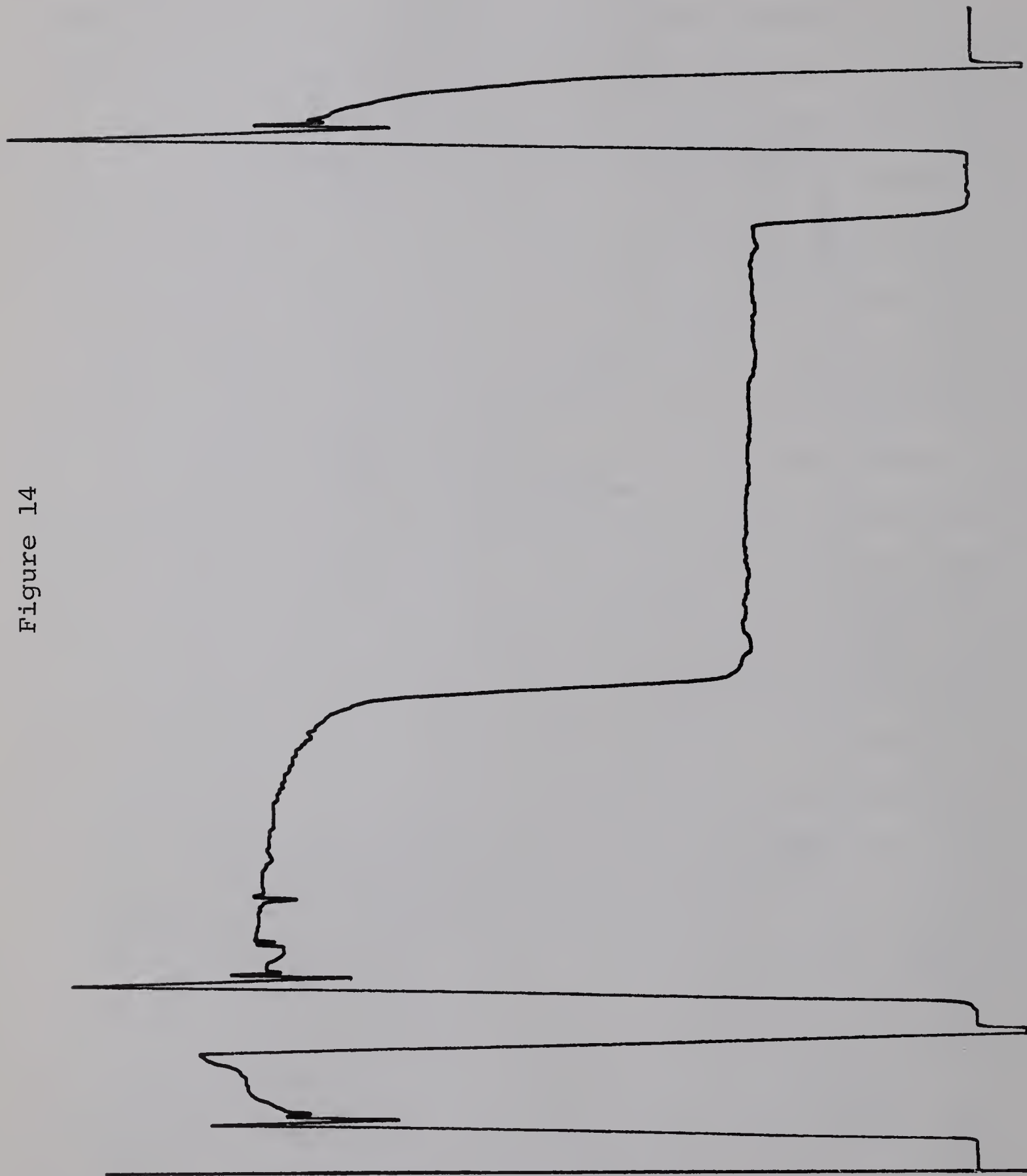


The sedimentation profile obtained by a preliminary resolution of uniformly labeled ^{14}C -nucleates, in a sucrose density-gradient. The sucrose density-gradient (5-25 %) was buffered with 0.01 M Tris-chloride-0.05 M sodium chloride, pH 7.4. The direction of sedimentation for the profile is from left to right in relation to the drawing, and the peaks behind the 16S component are DNA and sRNA.

solutions. For this reason, the ribosomal RNA components were precipitated from sodium chloride solution after their separation in sucrose density gradients.

Linear 5-25% sucrose-density gradients were prepared in 0.01 M Tris chloride-0.05 M sodium chloride, pH 7.4, according to the method of Kuff and Hymer (1966). One-half of the yield of nucleates (about 4 mg) from about 400 mg of E. coli cells was dissolved in 1 ml of the gradient buffer and layered onto a precooled 27 ml gradient, which was then centrifuged in the Beckman Model L ultracentrifuge at 4° and 24,000 r.p.m. for 15 hours in the SW-25 rotor. The rotor was allowed to decelerate without the brake. The bottom of the gradient tube was punctured and successive fractions, each containing 60 drops, were collected at 4°. A 20 µl sample was withdrawn from each fraction for determination of absorbance (260 mµ) and radioactivity (c.p.m.). The resolution by sucrose-density sedimentation is illustrated in Figure 13. The pooled fractions from each of the 16S and 23S peaks were mixed with an equal volume of cold isopropanol to precipitate the RNA (Stanley and Bock, 1965). After 8-12 hours at -20°, the precipitates were collected by centrifugation for 30 minutes at 4° and 14,000 x g. The RNA pellets were washed three times with cold isopropanol and ether, and after air-drying, the powders were stored at -20°.

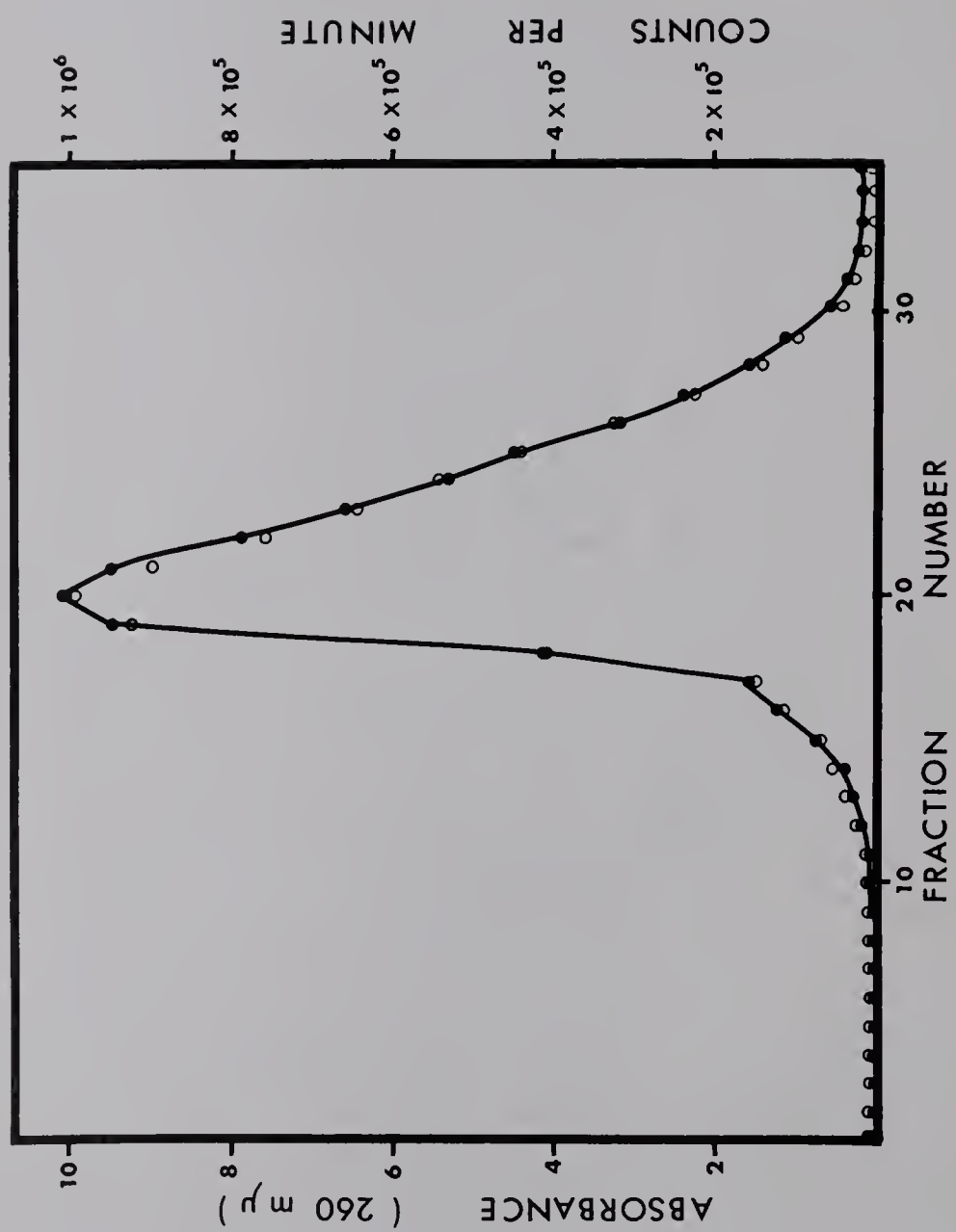
Figure 14



Legend of Figure 14

Densitometer trace of an ultraviolet photograph showing the sedimentation of 16S RNA in the analytical ultracentrifuge. The photograph was taken 16 minutes after reaching a speed of 59,780 r.p.m., in the Spinco Model E analytical ultracentrifuge. The sedimentation was performed at a concentration of 50 μ g of 16S RNA per milliliter of 0.1 M sodium chloride-0.01 M sodium acetate, pH 5.5.

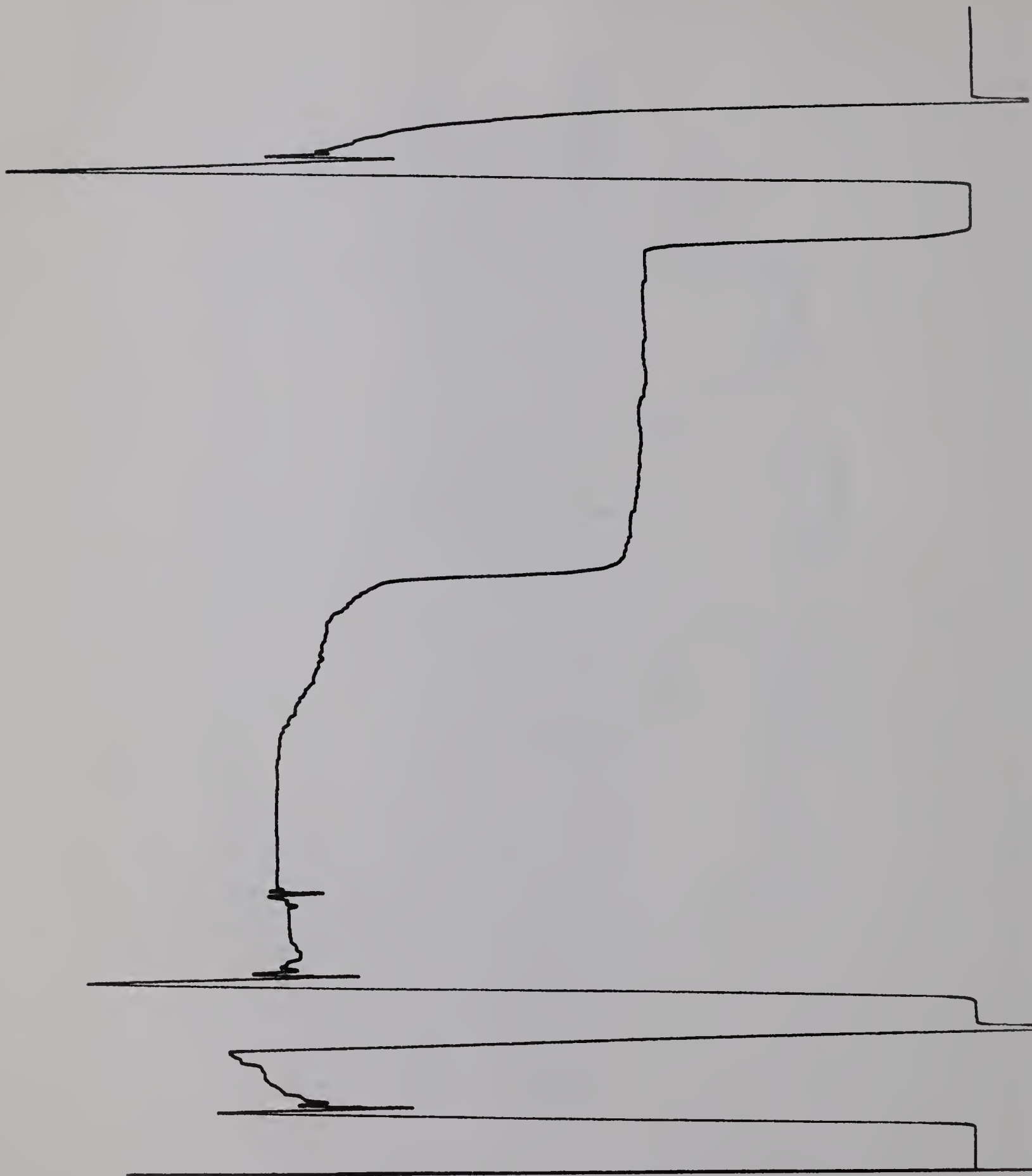
Figure 15



Legend of Figure 15

The sedimentation profile after a third passage of uniformly labeled ^{14}C -23S RNA through a sucrose density-gradient. The small trailing region was not included in the fractions pooled for analysis. The closed circles indicate absorbance and the open circles indicate radioactivity. The absorbance and radioactivity profiles are so nearly congruent that the curve for radioactivity has been omitted to simplify the diagram. Conditions for sedimentation have been described in the legend of Figure 13.

Figure 16



Legend of Figure 16

Densitometer trace of an ultraviolet photograph showing the sedimentation of 23S RNA in the analytical ultracentrifuge. The photograph was taken 16 minutes after reaching a speed of 59,780 r.p.m. in the Spinco Model E analytical ultracentrifuge. The sedimentation was performed at a concentration of 50 μ g of 23S RNA per milliliter of 0.1 M sodium chloride-0.01 M sodium acetate, pH 5.5.

The powder of 16S RNA was dissolved in 1 ml of distilled water and the solution was made 2 M with respect to sodium chloride. After 24 hours at 0°, the precipitate of 16S RNA was sedimented and washed three times with each of 70% ethanol, 95% ethanol, and ether. The air-dried powder was stored at -20°. The "16S RNA" gave a single sharply-sedimenting boundary in the analytical ultracentrifuge with an $S_{20,w}^0$ value of 16.2 (Figure 14).

The 23S RNA sample was contaminated with 16S RNA after only one sucrose density-gradient separation, and thus sucrose density-gradient centrifugation of the 23S RNA was repeated once or twice more. The sedimentation profile after a third centrifugation is illustrated in Figure 15. Fractions containing the 23S RNA were pooled, precipitated with isopropanol, dissolved in water, and precipitated from aqueous 2 M sodium chloride solution as described above. The precipitate of 23S RNA was washed with ethanol and ether, air-dried, and stored as a powder at -20°. The "23S RNA" gave a principal boundary in the analytical ultracentrifuge with an $S_{20,w}^0$ value of 22.8 (Figure 16).

(4) Preparation of Wheat Embryo rRNA.

Ninety g of commercial wheat embryo (Thatcher variety) was suspended in 600 ml of 0.05 M sodium

phosphate buffer (pH 6.8) and 600 ml of water-saturated phenol. The suspension was vigorously shaken for 15 minutes on a horizontal shaker at room temperature and centrifuged at $1500 \times g$ for 20 minutes at 0° . The upper aqueous layer was removed and extracted with ether to remove the phenol. Traces of ether were subsequently removed by aeration of the solution at 4° . The solution was then made 1 M with respect to sodium chloride by the addition of solid salt, and placed at 4° for 18 hours.

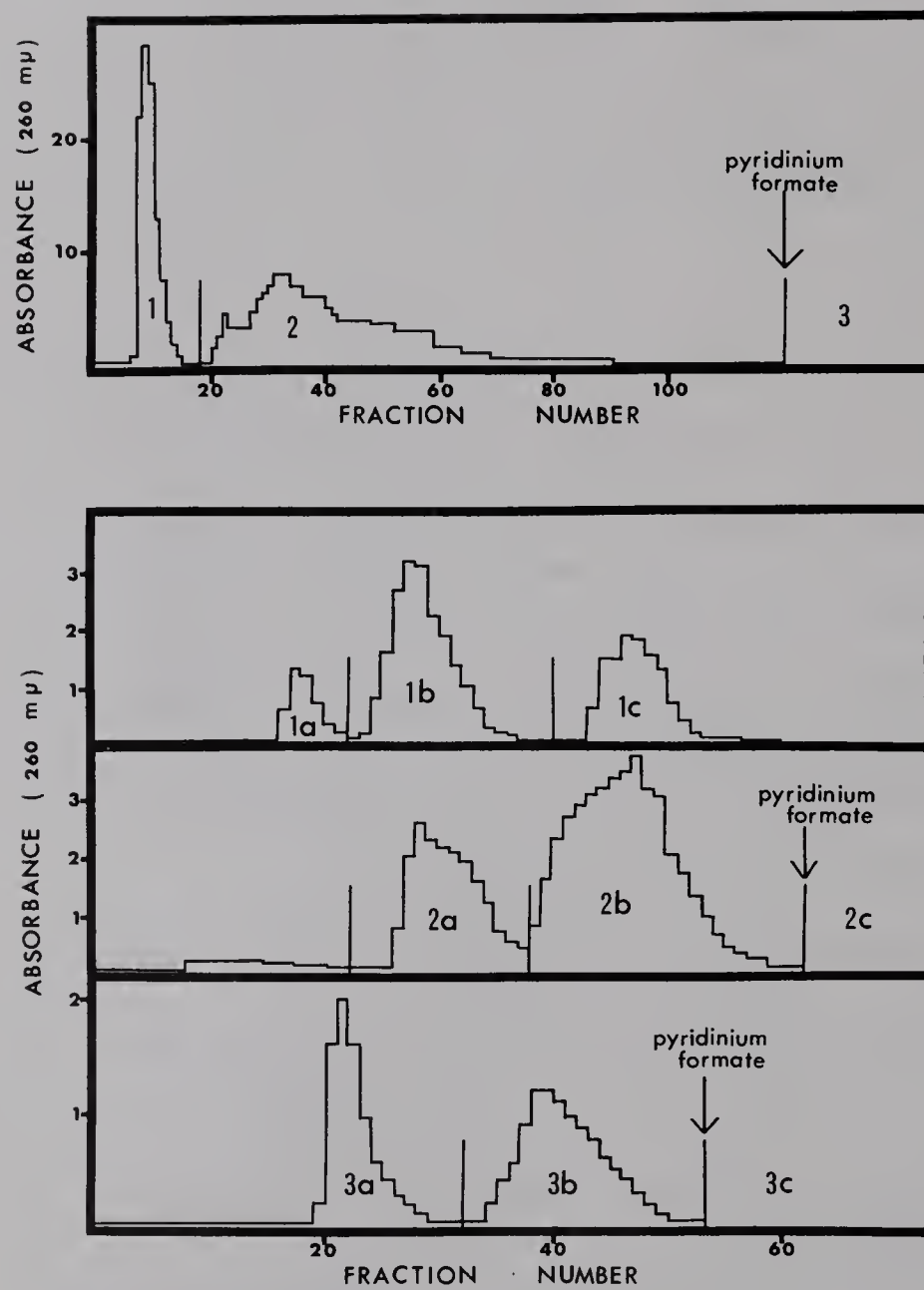
The rRNA was collected by centrifugation at 0° and the resulting pellet was washed three times with each of 70% ethanol, 95% ethanol, and ether. The air-dried powder of rRNA was subjected twice more to precipitation from 1 M sodium chloride, followed by washing with ethanol and ether, and air-drying. The final powder (1 g) was stored at -20° .

An aqueous solution of the wheat embryo rRNA had an extinction coefficient (1%, 1 cm) of 220-250 at 260 m μ . By analytical ultracentrifugation, the wheat embryo rRNA was shown to be comprised of two sharply-sedimenting boundaries with $S_{20,w}^0$ values of 17.9 and 25.6.

(5) Preparation of Carrier Alkali-Stable Dinucleotides From Wheat Embryo rRNA.

Six grams of purified wheat embryo rRNA was hydrolyzed in 1 M alkali according to the procedure

Figure 17



Legend of Figure 17

Elution profiles for the fractionation of alkali-stable dinucleotides of wheat embryo rRNA. The dinucleotides were initially fractionated into three groups (Pools 1, 2, and 3) by chromatography at pH 2.0 on DEAE-cellulose (top figure). Each of these pools was sub-fractionated into three fractions by chromatography at pH 4.6 on DEAE-cellulose. The fractions contained: (1a) CxpCp; (1b) AxpCp, CxpAp; (1c) AxpAp; (2a) UxpCp, CxpUp; (2b) AxpUp, UxpAp, GxpCp, CxpGp; (2c) GxpAp, AxpGp; (3a) UxpUp; (3b) UxpGp, GxpUp; and (3c) GxpGp.

described in Part I for E. coli RNA. After neutralization, the hydrolyzate was divided into two parts, and each part was fractionated on a 4.5 cm x 30 cm DEAE-cellulose column (formate form) according to the procedures already described (Part I). After desalting on DEAE-cellulose columns, the dinucleotide fractions obtained from each of the two columns were combined.

The combined dinucleotide fraction was applied to a 2.5 cm x 20 cm DEAE-cellulose column. Elution was begun with 1 M formic acid and 10 ml fractions were collected and monitored for absorbancy at 260 m μ . Two peaks were obtained in the elution profile, and, following the complete elution of the second peak, the remaining material was eluted with 1 M pyridinium formate, pH 4.5. The elution profile of material from this column is presented in Figure 17.

The fractions comprising each of the first two peaks were separately pooled (Pools 1 and 2), neutralized with pyridine, evaporated to dryness, and re-evaporated from 0.5 M ammonium hydroxide solution. The material eluted from the column with 1 M pyridinium formate (Pool 3) was also evaporated to dryness and re-evaporated from ammonium hydroxide solution.

Each of the three fractions obtained in this way (Pools 1, 2, and 3) was further fractionated by chromato-

graphy on 2.5 cm x 20 cm DEAE-cellulose columns. In each case the eluent employed was 0.1 M sodium acetate, pH 4.6 (Lane and Allen, 1961). Thirty ml fractions were collected. Each subfraction was pooled and desalted on DEAE-cellulose columns. The elution profiles for each of these columns is presented in Figure 17. As was expected from earlier studies (Singh and Lane, 1964a), Pool 1 contained (1a) CxpCp, (1b) AxpCp, CxpAp and (1c) AxpAp; Pool 2 contained (2a) UxpCp, CxpUp, (2b) AxpUp, UxpAp, GxpCp, CxpGp and (2c) GxpAp, AxpGp; Pool 3 contained (3a) UxpUp, (3b) UxpGp, GxpUp and (3c) GxpGp.

Each of the resulting twelve fractions was chromatographed in the two-dimensional paper chromatographic system and determined to be free of ultraviolet-absorbing contaminants. It was now possible to reconstitute the resolved dinucleotides in any desired proportions. In particular, it was possible to omit UxpCp-CxpUp from the reconstituted mixture and replace it by N⁴MeCxpCp in the case of 16S RNA hydrolyzates (see below).

(6) Fractionation of Alkali Hydrolyzates of Uniformly ¹⁴C-labeled 16S and 23S E. coli RNA.

About 1-2 mg of uniformly labeled ¹⁴C-RNA was dissolved in 100 μ l of water and the solution was made 1 M with respect to sodium hydroxide by the addition of 25 μ l of 5 M sodium hydroxide solution. The solution

was allowed to stand at room temperature for 90 hours before dilution to about 50 ml, and neutralization with formic acid. Carrier nucleosides (0.25 μ mole of each of A, G, C, and U), nucleoside diphosphates (0.25 μ mole of each of pAp, pGp, pCp, and pUp) and dinucleotides were added to the neutralized hydrolyzate and the volume was made up to 250 ml. About 2 μ moles of total dinucleotides were added to the hydrolyzate and they were present in the proportions found in wheat embryo rRNA. Carrier nucleoside monophosphates were unnecessary since the hydrolyzates contained 4-5 μ moles of these compounds from the hydrolysis of the E. coli RNA. Hydrolyzates were fractionated on 2.5 cm x 10 cm columns of DEAE-cellulose by the stepwise elution scheme described in Part I. The dinucleotides UxpCp-CxpUp were replaced by N⁴MeCxpCp (0.3 μ mole) in carrier dinucleotides added to neutralized hydrolyzates of 16S RNA.

N⁴MeCxpCp was prepared from E. coli rRNA according to the procedure outlined in Part I of the thesis.

(7) Resolution of Alkali-stable Dinucleoside Phosphates From Uniformly Labeled ¹⁴C-rRNA of E. coli.

The dinucleotides eluted from DEAE-cellulose columns were desalted and treated with E. coli phosphomonoesterase in order to convert them to dinucleoside phosphates as described in Part I. The dinucleoside

TABLE VIII

Efficiency of Counting ^{14}C -labeled Nucleate Derivatives
Under Various Conditions

Sample*	Counting Efficiency†
1 ml aqueous solution	70-75%
1 ml acidic eluent from a one-dimensional chromatogram (chromatographic system A)	60-70%
1 ml acidic eluent from a two-dimensional chromatogram (chromatographic system B)	60-70%
Paper strip from a one-dimensional chromatogram (chromatographic system A)	50-60%
Paper strip from a two-dimensional chromatogram (chromatographic system B)	40-50%

* Radioactivity measurements were performed using a Nuclear Chicago liquid scintillation counter (Model 8260). Samples were added to 10 ml of Bray's solution (Bray, 1960). Uniformly labeled ^{14}C -N, ^{14}C -Np, and ^{14}C -pNp were tested, and only in the case of direct radioactivity measurements of ^{14}C -N-containing paper strips (where the nucleosides eluted from the paper) were there any differences observed among the counting efficiencies for the different types of compounds. A time-dependent increase to a counting efficiency of about 70% was observed for nucleosides.

† Relative to the value obtained when the same amount of material was dissolved in 25 μl of water, and added to 10 ml of Bray's solution. The absolute counting efficiency for nucleotides, dissolved in 25 μl of water and added to 10 ml of Bray's solution, was 70%.

phosphates were then desalted on a 4.5 cm x 10 cm DEAE-cellulose column before application to paper for two-dimensional chromatography. Small amounts of Ap and Gp sometimes trailed during column separations on DEAE-cellulose, and became included in the dinucleotide fraction, but these were converted to A and G by phosphomonoesterase treatment, and removed when the dinucleoside phosphates were desalted on DEAE-cellulose.

Areas corresponding to individual dinucleoside phosphates were located on paper chromatograms by the ultraviolet quenching of the carrier compounds, excised, and placed in 10 ml of scintillation fluid (Bray, 1960) for counting in a Nuclear Chicago liquid scintillation counter. The remainder of the chromatogram was sectioned into areas of roughly uniform size, and counted. The efficiency of counting paper strips was found to be 40% and appropriate correction could be made to relate the radioactivity of individual areas to the radioactivity in the initial hydrolyzate (Table VIII).

(8) Characterization of Individual Dinucleoside Phosphates from Uniformly Labeled ^{14}C -rRNA of *E. coli*.

The dinucleoside phosphates did not elute into the scintillation fluid, and after paper strips had been counted, they could be removed from scintillation vials without loss of dinucleoside phosphate from the paper.

The papers were extracted three times with 20 ml of ether before the scintillation fluid had evaporated. Ether-extraction removed the components of the scintillation fluid, and the dinucleoside phosphates could be eluted, and their ultraviolet absorption spectra measured, without evidence of contamination from ultraviolet-absorbing impurities. For this purpose, it was essential to perform the ether-extraction before the scintillation fluid had evaporated, otherwise the extraction did not efficiently remove naphthalene from the papers. The dinucleoside phosphates were eluted from the ether-extracted paper with water and then desalted using charcoal discs as described in Part I of the thesis.

Individual dinucleoside phosphates were characterized by hydrolysis with snake venom, to yield the constituent nucleosides and 2'-O-methyl nucleosides. Dinucleoside phosphates were dissolved in 25 μ l of water and mixed with 25 μ l of 1 M ammonium formate buffer, pH 9.2, and 50 μ l of a 0.3% solution of Russell viper venom. The digests were incubated at 37^o for 24 hours and then desalted on charcoal discs before resolution of hydrolysis products.

Aliquots of each digest were separated by paper electrophoresis in tetraborate buffer, pH 9.2 and also by chromatography on sulphate-impregnated paper (Part I),

with standard marker compounds of the four nucleosides and their 2'-O-methyl derivatives. The electrophoretic and chromatographic systems provided a useful set of complementary separation patterns, since the electrophoretic system caused the normal nucleosides to migrate ahead of the 2'-O-methyl nucleosides, whereas the chromatographic system caused the 2'-O-methyl nucleosides to migrate well ahead of the corresponding normal nucleosides.

(9) Pseudouridylate Analyses of Uniformly Labeled ^{14}C -16S RNA and ^{14}C -23S RNA of *E. coli*.

Analyses for pseudouridylate in uniformly labeled ^{14}C -16S RNA and uniformly labeled ^{14}C -23S RNA were performed by direct two-dimensional paper chromatography of a neutralized alkali hydrolyzate containing about 1×10^6 c.p.m. and 0.5 μmole of carrier pseudouridylate. The pseudouridylate was eluted from the chromatogram, desalted by charcoal adsorption, and converted to pseudouridine by treatment with *E. coli* phosphomonoesterase. The pseudouridine was chromatographed once more in the same two-dimensional paper chromatographic system, and all areas of the chromatogram were eluted and counted. The pseudouridine area was free from any surrounding background of radioactivity, and the measured radioactivity, in conjunction with the recovery of carrier pseudouridylate, could be used to obtain a minimal estimate for the trace amount of pseudouridylate in each of the 16S and 23S components.

TABLE IX

Distribution of Radioactivity Among the Products Obtained by Fractionation of an Alkali Hydrolyzate of 23S RNA on DEAE-cellulose

Fraction*	^{14}C -radioactivity (c.p.m.)
N	2.2×10^3
Np	3.0×10^6
NxpNp	2.5×10^4
pNp	2.3×10^3

* As indicated in the text, the fractionation on DEAE-cellulose was monitored by the employment of ultra-violet-absorbing carriers except in the case of the Np-fraction, where the quantity of these compounds derived from the 23S RNA was sufficient for detection without carrier. The charging solution contained 3.2×10^6 c.p.m. and the radioactivity measurements of the various fractions were made on aliquots of the material recovered from each fraction, after desalting. With allowance for 6-8% deamination of Cp \rightarrow Up during the 90 hour hydrolysis in 1 M alkali at room temperature, the proportions of the major nucleotides were in agreement with published values (Stanley and Bock, 1965) for E. coli 23S RNA, and more than 99% of the radioactivity was confined to the four principal nucleotides after paper chromatography in two dimensions.

(iii) Results of Analyses

(1) Alkali-Stable Dinucleotide Sequences in Uniformly Labeled ^{14}C -16S RNA and ^{14}C -23S RNA of *E. coli*.

The distribution of radioactivity among the different ion-exchange fractions of an alkali hydrolyzate of uniformly labeled ^{14}C -23S RNA from *E. coli* is shown in Table IX. The results of analyses of the nucleoside and nucleoside diphosphate fractions will be presented in Part V of the thesis. The nucleoside monophosphates were desalted and separated by paper chromatography to assess their specific activities. The specific activity of the nucleotides from different preparations of RNA was always in the range 8×10^5 to 9×10^5 c.p.m. per μmole of nucleotide, and for any given preparation, the measured specific activities of the purine nucleotides were within 2% of one another, and exceeded the specific activities of the pyrimidine nucleotides by 10%, as expected for uniform ^{14}C -labeling.

The bulk of the counts from the dinucleotide fraction have not been characterized; however, the uncharacterized material did not interfere with the present analyses since it was well separated by two-dimensional paper chromatography from the compounds which were of interest for the present study. Thus, when the dinucleotides were converted to dinucleoside phosphates and

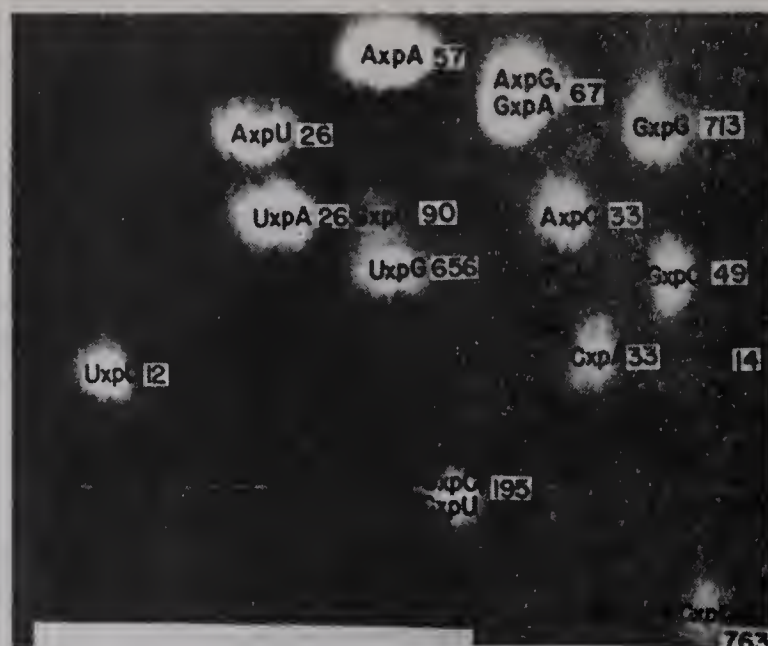


Figure 18

An ultraviolet contact photograph depicting the two-dimensional paper chromatographic resolution of carrier dinucleoside phosphates. Two-dimensional chromatography was effected with system A in the first dimension (developed right to left in relation to the photograph), and with system B in the second-dimension (developed top to bottom in relation to the photograph). The numbers to the right of the symbols for the dinucleoside phosphates indicate the radioactivity (c.p.m.) measured for each of the excised spots in a typical experiment in which 5×10^6 c.p.m. of 23S RNA was subjected to alkali hydrolysis. The white band at the bottom of the photograph has been affixed to indicate the beginning of the area which contains uncharacterized radioactive material (about 10^4 c.p.m.). $N^4\text{MeCxpC}$ occupies a position adjacent to UxpC-CxpU, where the number 195 has been drawn.

TABLE X

The Distribution of Radioactivity Among the Carrier Dinucleoside Phosphates in Experiments with E. coli 23S RNA

Dinucleoside Phosphate	¹⁴ C radioactivity (c.p.m.)	% of Total Counts
AxpA	52	2.1
AxpG*	86	3.5
AxpC	34	1.4
AxpU	27	1.1
GxpA*	86	3.5
GxpG	641	26.1
GxpC	59	2.4
GxpU	64	2.6
CxpA	27	1.1
CxpG	12	0.5
CxpC	631	25.7
CxpU*	171	7.0
UxpA	12	0.5
UxpG	638	26.0
UxpC*	171	7.0
UxpU	5	0.2

* The isomeric pairs AxpG-GxpA and UxpC-CxpU were not resolved by paper chromatography, and hence the entry for each member of an unresolved pair includes the contribution from the other isomer. Therefore, the values for each member of an unresolved pair are maximal estimates, and the total of all values in the table will be 100% when the value for one of each of the isomers of an unresolved pair is included in the summation.

In different experiments the background count for areas of the chromatogram comparable in size to the area occupied by an individual dinucleoside phosphate corresponded to 0.8-1.2% of the total counts found in all dinucleoside phosphates. Therefore, the radioactivity found in spots other than GxpG, CxpC, UxpG and CxpU-UxpC is of questionable significance.

resolved by two-dimensional chromatography, the uncharacterized material moved well ahead of all of the dinucleoside phosphates in the second dimensional development. An ultraviolet contact photograph showing the two-dimensional paper chromatographic resolution of carrier dinucleoside phosphates is shown in Figure 18, and the beginning of the area which contains the uncharacterized material has been indicated. The numbers which appear to the right of the symbols for the dinucleoside phosphates in Figure 18 illustrate the amount of ^{14}C -label found in each of the excised spots, in a typical experiment with uniformly labeled ^{14}C -23S RNA from E. coli. The uncharacterized radioactive material was also found with hydrolyzates of 16S RNA and was, unlike the dinucleoside phosphates, readily dissolved in the scintillation fluid.

The ^{14}C -label from the dinucleotide fraction of the alkali hydrolyzates of three preparations of 23S RNA was distributed among the carrier dinucleoside phosphates in the mean proportions shown in Table X. The most prominent dinucleoside phosphates, GxpG, CxpC, and UxpG were further characterized by hydrolysis with whole snake venom to produce equimolar amounts of the normal and 2'-O-methyl nucleosides. Prior to hydrolysis, a further 0.3 μmole of the carrier dinucleoside phosphates was added, to

Figure 19

	1	2	3	4
ORIGINS →	7	5	0	26
	5	6	8	17
	0		0	25
	6	1	0	28
	1	5	4	8
	5		7	11
		6	8	13
		6		11
	2			
	2		1	
	4		2	
	0	15	5	7
			0	28
			2	20
	9	6	7	
	4	12	4	
			0	
	1	7	3	14
	3		2	31
		1	1	
		11		
	1			10
				18
				9

C

G

A

U

C
149

G
212

C
203

G
118

CX
175

GX
139

Ux
253

N⁴Me
-CX-257

Legend of Figure 19

The distribution of radioactivity among the carrier nucleosides and 2'-O-methyl nucleosides after paper chromatography of the products formed by the snake venom hydrolysis of alkali-stable dinucleoside phosphates. An aliquot of the whole venom digest of each dinucleoside phosphate was chromatographed in system A, to resolve the nucleosides and 2'-O-methyl nucleosides. Following the location of each of the carrier nucleosides by ultraviolet-quenching, areas of the paper were excised, and placed in scintillation fluid for radioactivity measurements. The numbers 1, 2, 3, and 4 at the top of the figure, indicate the hydrolyzates of CxpC, UxpG, N⁴MeCxpC, and GxpG, respectively. The migration of marker nucleosides is indicated at the left of the figure. The resolution of the nucleoside, N⁴MeCx, differs from that illustrated in Figure 9 because the hydrolyzate, in this experiment, was not desalted prior to chromatography, and therefore contained a small amount of ammonium formate buffer, used in buffering venom digests. The presence of this buffer is responsible for the increased R_f value observed for N⁴MeCx. It is not known why the radioactivity in non-carrier areas of the GxpG hydrolyzate is high, but it is thought that this is probably a result of a secondary interaction with the components in the scintillation fluid prior to hydrolysis of the dinucleoside phosphate.

Figure 20

1	2	3	
0	0	0	
0	1	0	
0	0	0	
0	3	0	
6	0	3	
⁴ NM _E C _X ⁹⁹	3	C _X ⁶⁰	⁴ NM _E C _X , C _X , A _X
0	1	2	
2	U _X 54	0	G _X , U _X
0	4	0	
0	13	1	
1	1	0	
C 91	1	C 56	C, A
1	3	5	
6	G 77	1	G, U
1	5	4	
2	5	0	
0	1	0	
3	1	0	
1	0	0	

← ORIGIN

+

Legend of Figure 20

The distribution of radioactivity among carrier nucleosides and 2'-O-methyl nucleosides after paper electrophoresis of products formed by snake venom hydrolysis of alkali-stable dinucleoside phosphates. Paper electrophoresis was performed in sodium tetraborate buffer, pH 9.2. Following detection of the carrier nucleosides and 2'-O-methyl nucleosides, by ultraviolet quenching, each of the areas indicated in the figure, was excised and placed in scintillation fluid for radioactivity measurements. The numbers indicate the radioactivity (c.p.m.) obtained for each of the excised areas. The numbers 1, 2, and 3, at the top of the figure, indicate the hydrolyzates of N⁴MeCxpC, UxpG, and CxpC, respectively. The migration of marker nucleosides is indicated at the right of the figure.

ensure detection of the hydrolysis products by their ultraviolet quenching after paper chromatographic or electrophoretic separation. The hydrolysis products were separated by both paper electrophoresis and paper chromatography, and the ^{14}C -label was coincident with the expected ultraviolet-absorbing carrier in each case. The distribution of ^{14}C -label among carrier nucleosides and 2'-O-methyl nucleosides, following chromatography, is illustrated in Figure 19. Similar experiments, involving electrophoretic separations, are illustrated in Figure 20.

Because wheat embryo rRNA contains about 20 times more 2'-O-methyl ribose than does E. coli rRNA, it is a convenient source from which to prepare large amounts of authentic carrier dinucleotides. The usefulness of wheat embryo rRNA as a source of carrier dinucleotides is further enhanced by the fact that it yields all sixteen alkali-stable dinucleotides of the type NxpNp in which N is any one of the four major ribonucleosides and Nx is the 2'-O-methyl derivative of any one of these nucleosides. However, as pointed out in Part I, the rRNA from E. coli contains an unusual alkali-stable dinucleotide sequence, $\text{N}^4\text{MeCxpCp}$. Thus, when carrier dinucleotides from wheat embryo were used with alkali hydrolyzates of 16S RNA from E. coli, it

TABLE XI

The Distribution of Radioactivity Among the Carrier Dinucleoside Phosphates in Experiments with E. coli 16S RNA

Dinucleoside Phosphate	¹⁴ C-radioactivity (c.p.m.)	% of Total Counts
AxpA	31	1.8
AxpG*	79	4.6
AxpC	21	1.2
AxpU	17	1.0
GxpA*	79	4.6
GxpG	120	7.0
GxpC	29	1.7
GxpU	14	0.8
CxpA	22	1.3
CxpG	32	1.9
CxpC	105	6.1
CxpU*	7	0.4
UxpA	26	1.5
UxpG	93	5.4
UxpC*	7	0.4
UxpU	10	0.6
N ⁴ MeCxpC	1106	64.5

* See the legend of Table X concerning the entries for unresolved isomers. For reasons noted in the legend of Table X, values in the neighbourhood of 1-2% are of questionable significance. Most of the radioactivity that is significantly above background (excluding N⁴MeCxpC) is found in the sequences CxpC, GxpG, and UxpG, which are the principal sequences in the 23S RNA.

was found that an area of the chromatogram, overlapping the unresolved isomeric dinucleoside phosphates, UxpC-CxpU, contained more ^{14}C -label than was found in all other dinucleoside phosphates combined. The ^{14}C -label had precisely the same R_f value as UxpC-CxpU in the second dimension but moved slightly behind UxpC-CxpU in the first dimension; consequently, the ^{14}C -label traveled as a discrete, compact spot, overlapping, but not coincident with UxpC-CxpU (see Figure 18 and caption). The compound previously characterized as $\text{N}^4\text{MeCxpCp}$, was prepared from 4 g of E. coli rRNA (Part I) and, as expected, it overlapped CxpUp-UxpCp when the compounds were co-chromatographed in two dimensions. In subsequent experiments, CxpUp-UxpCp, were replaced by $\text{N}^4\text{MeCxpCp}$ in the carrier dinucleotides added to alkali hydrolyzates of 16S RNA. The bulk of the label in the dinucleoside phosphates co-chromatographed precisely with N^4MeCxpC during two-dimensional chromatography. The ^{14}C -label from the dinucleotide fraction of alkali hydrolyzates of two 16S RNA preparations was distributed among the carrier dinucleoside phosphates in the mean proportions shown in Table XI.

When the material in the N^4MeCxpC spot of two-dimensional chromatograms was treated with snake venom, one-half of the ^{14}C -label migrated coincidentally with

N^4 MeCx during both borate electrophoresis and chromatography on sulphate-impregnated paper, whereas the other half of the label migrated with normal cytidine during both electrophoresis and chromatography. These chromatographic and electrophoretic separations are illustrated in Figures 19 and 20.

It is worth noting that the N^4 MeCx derived from uniformly-labeled N^4 MeCxpC contained 10-20% more radioactivity than the cytidine derived from the same compound, as would be expected for two additional methyl groups in N^4 MeCx relative to C. Experiments in Part III on the incorporation of methyl- 14 C groups from methionine into the rRNA of E. coli, provide further evidence on this point.

In connection with efforts to establish the maximum amount of N^4 MeCxpCp which could be present in 23S RNA, the material in the UxpC-CxpU- N^4 MeCxpC areas of three two-dimensional chromatograms was eluted, desalted and treated with whole venom. Analysis of the hydrolysis products indicated that the starting material was chiefly UxpC and CxpU and that the amount of N^4 MeCxpC did not exceed 2% of the quantity found in 16S RNA. Even after repeated sucrose density gradient centrifugations, the 23S RNA showed evidence of contamination by a small quantity of 16S RNA (see Figure 16), and it is concluded

that any $N^4\text{MeCxpC}$ found in 23S RNA probably arose from contaminant 16S RNA. After correction for deamination of CxpCp during the 90 hour period in 1 M alkali, the combined amounts of UxpCp and CxpUp in 23S RNA could not exceed 15% of the quantity of the CxpCp sequence.

(2) Pseudouridylate Analyses of Uniformly Labeled ^{14}C -16S RNA and ^{14}C -23S RNA of *E. coli*.

The quantity of pseudouridylate was found to be 0.06 mole % in 16S RNA and 0.15 mole % in 23S RNA. The preparations of 16S and 23S RNA, that were examined for pseudouridylate content, derived from the same uniformly labeled ^{14}C -rRNA preparation. These quantities are somewhat lower than expected from the analysis of large-scale rRNA preparations by spectral methods (Part I of the thesis), but, in agreement with the observations of Dubin and Günalp (1967), 23S RNA contained about twice as much pseudouridylate as did 16S RNA, although Dubin and Günalp found larger absolute amounts of pseudouridylate in each of the components.

(iv) Discussion

Although the aims of the experiments described in this part of the thesis were chiefly qualitative in nature, it is perhaps interesting to examine some of the quantitative features of the available data. After making substantial corrections for quenching of radio-

activity on two-dimensional paper chromatograms (about 60%), and for recovery of carrier compounds from alkali hydrolyzates (about 60%), it is possible to conclude that GxpGp, CxpCp, and UxpGp accounted for 2971 c.p.m., 3179 c.p.m., and 2733 c.p.m., respectively, of a total of 5.0×10^6 c.p.m. in an alkali hydrolyzate of 23S RNA. This would mean that there is 0.030 mole % Gx, 0.031 mole % Cx, and 0.028 mole % Ux in 23S RNA. Similarly, $N^4\text{MeCxpCp}$ contained 4608 c.p.m. of a total of 3.0×10^6 c.p.m. in an alkali hydrolyzate of 16S RNA, and this would indicate that there is 0.077 mole % of $N^4\text{MeCx}$ in 16S RNA. Consequently, each of the 2'-O-methyl nucleotides would be present in roughly equal molar amount in an rRNA preparation composed of 33% 16S RNA, and 67% 23S RNA, on a weight basis. Alumina-grinding of E. coli yields rRNA preparations that contain about 33% 16S RNA, and 67% 23S RNA, and thus Gx, Cx, Ux, and $N^4\text{MeCx}$ would each be expected to account for roughly 25% of the total 2'-O-methyl nucleosides in such preparations, and this was found to be the case in the experiments using methyl- ^{14}C precursor, described in Part III of the thesis.

The proportions found for the large-scale rRNA preparation, analyzed in Part I of the thesis, were 38% $N^4\text{MeCx}$, 18% Gx, 24% Cx, and 17% Ux (Table III). The large-scale preparation of E. coli rRNA, analyzed in

Part I of the thesis, was obtained by aqueous phenol-extraction of whole cells, a method known to yield rRNA enriched in 16S RNA and depleted in 23S RNA (Artman, Fry, and Engelberg, 1966). The end group data for 16S RNA and 23S RNA in Part V of this thesis, can be used in conjunction with end group data for the large-scale rRNA preparation analyzed in Part I of the thesis (Lane, 1962), to arrive at the conclusion that the large-scale preparation was enriched in 16S RNA and depleted in 23S RNA. Thus, the higher proportion of $N^4\text{MeCx}$, relative to the other 2'-O-methyl nucleosides in the large-scale rRNA preparation is not surprising since the preparation was probably enriched with 16S RNA, at the expense of 23S RNA. For equal weight percentages of 16S RNA and 23S RNA in the large-scale rRNA preparation analyzed in Part I of the thesis, it could be concluded from the data in Table III, that there would be 0.040 mole % Gx, 0.052 mole % Cx, 0.036 mole % Ux in 23S RNA, and 0.082 mole % $N^4\text{MeCx}$ in 16S RNA. The value for $N^4\text{MeCx}$, obtained in this manner, is in good agreement with the value obtained for uniformly labeled ^{14}C -16S RNA, while the values for Gx, Cx, and Ux are somewhat greater than obtained for uniformly labeled ^{14}C -23S RNA.

A component which occurs with a frequency of 1 nucleotide residue per polynucleotide chain in 23S RNA,

accounts for 0.05 mole % of the component nucleotides, and a component which occurs with a frequency of one residue per polynucleotide chain in 16S RNA, accounts for 0.08 mole % of the component nucleotides. Although definitive conclusions are obviously impossible, it would appear that each of Gx, Cx, and Ux is present in an amount corresponding to 0.5 to 1 residue per polynucleotide chain in 23S RNA, and N⁴MeCx is present in an amount approaching one residue per polynucleotide chain in 16S RNA.

Additional evidence will be required to establish whether the much smaller amounts of radioactivity found in other carrier dinucleoside phosphates is significant, but it is clear that other sequences, if present, are confined to a relatively small proportion of the total polynucleotide chains in the 16S and 23S fractions of rRNA. Evidence that the sequences CxpUp and UxpCp may be present in small amounts, in 23S RNA, has been obtained in this present study. Further evidence for the possible occurrence of relatively small amounts of additional alkali-stable dinucleotide sequences, comes from the studies of Part I, where faint ultraviolet-quenching areas were detected in areas of two-dimensional paper chromatograms that could be occupied by dinucleoside phosphates. It is apparent that further studies along these lines could provide useful information about the hetero-

geneity of rRNA.

The parallel noted between pseudouridylate and 2'-O-methyl nucleotides in the large-scale preparation of Part I of the thesis, is also evident from the studies in this section of the thesis, since a larger quantity of pseudouridylate in 23S RNA is associated with a larger amount of 2'-O-methyl nucleotide, relative to the smaller amounts of both of these components in 16S RNA.

Part III. The in vivo Incorporation of Methyl Groups into the Ribose of Escherichia coli Ribosomal RNA

(i) Introduction

The in vivo incorporation of methyl groups into RNA was first shown by Mandel and Borek (1963), who analyzed RNA isolated from E. coli grown on a medium containing (methyl- ^{14}C)-methionine, and found that the major nucleotides were virtually devoid of radioactivity whereas the minor methyl-substituted nucleotides were heavily labeled. These observations were extended by Starr and Fefferman (1964), who, using an otherwise similar approach, resolved the radioactive RNA into sRNA and rRNA, by sucrose density-gradient centrifugation, and thereby showed that the methyl groups from an exogenous supply of methionine were efficiently utilized by E. coli for in vivo methylation of both sRNA, and to a lesser degree, rRNA.

In the studies just described, the in vivo enzymic methylations of E. coli sRNA and rRNA were shown to have resulted in the methylation of the heterocycles of the four major nucleotide constituents in RNA. However, E. coli sRNA (Hall, 1964; Nichols and Lane, 1966) and E. coli rRNA (Nichols and Lane, 1966) also contain methyl-substituents in the glycosyl parts of the major nucleotides, and so the studies in this part of the thesis were undertaken in order to establish if the methyl groups from an

exogenous supply of (methyl- ^{14}C)-methionine would also be utilized efficiently for in vivo 2'-O-methylation of E. coli RNA. Sanger, Brownlee, and Barrell (1965) provided evidence that the methyl group from an exogenous supply of (methyl- ^{14}C)-methionine was efficiently incorporated into the GxpG sequence of E. coli sRNA. The present study was directed toward an examination of E. coli rRNA, in the hope that there would be an equally efficient utilization of exogenous (methyl- ^{14}C)-methionine for in vivo methylation of both the N^4 - and 2'-O- positions in N^4 ,2'-O-dimethyl cytidine. If this were so, it would be possible to obtain confirmatory evidence of structure for the novel component $\text{N}^4\text{MeCxpCp}$, since it would have a methyl- $^{14}\text{C}/^{32}\text{P}$ -ratio twice as great as that found for GxpGp, CxpCp and UxpGp.

The addition of chloramphenicol to cultures of bacteria, sensitive to this antibiotic, causes an inhibition of protein synthesis while RNA synthesis continues. The high molecular weight RNA that is formed in the presence of chloramphenicol (CM-rRNA) is believed to be a precursor of normal rRNA, and is present in ribonucleoprotein particles (CM-particles), which are thought to be precursors of the normal 70S ribosomes. Dubin and Elkort (1964) suggested that abnormal sedimentation properties of CM-rRNA, relative to normal rRNA, might

relate to conformational differences between the two, and that the conformational difference may arise from an "undermethylation" of CM-rRNA. This view was experimentally supported by the work of Gordon, Boman and Isaksson (1964), who showed that the component CM-rRNA would accept methyl-¹⁴C when CM-particles were incubated with (methyl-¹⁴C)-S-adenosylmethionine, in vitro. Particles, similar to CM-particles, accumulate during methionine-deprivation of a methionine-dependent auxotroph of E. coli, and they are termed methionine-starvation particles, or MS-particles. As in the case of CM-sensitive bacteria, RNA synthesis continues during methionine-deprivation and this is termed a "relaxed" control of RNA synthesis, and the mutant is therefore referred to as a "relaxed" mutant. The high molecular weight RNA formed under these conditions of "relaxed" control (MS-rRNA) is also "undermethylated" (Gordon and Boman, 1964), and is present in the MS-particles. Nofal and Srinivasan (1966) have observed that in vivo methylation of MS-RNA, upon the addition of (methyl-¹⁴C)-methionine to a methionine-starved culture, occurs prior to "re-arrangement" of MS-particles to give normal 70S ribosomes. Thus, it would appear that in vivo methylation of "undermethylated" rRNA precursors, may be an important step for potentiating the in vivo synthesis of ribosomes.

In the studies just described, the process of methylation was visualized wholly in terms of the methyla-

tion of heterocycles since the occurrence of methyl-substituted heterocycles in E. coli rRNA had been established (Starr and Fefferman, 1964). With the discovery that there was a minute quantity of 2'-O-methyl ribosyl components in E. coli rRNA, it was of interest to examine the manner in which their in vivo methylation might be affected by chloramphenicol, in CM-sensitive bacteria, and by methionine-deprivation of a "relaxed" mutant. The studies of the effects of chloramphenicol on 2'-O-methylation in chloramphenicol-sensitive bacteria will be described in this part of the thesis, but the study of a "relaxed" mutant will be considered in a separate part of the thesis (Part IV), where in vitro incorporation of methyl-¹⁴C into E. coli rRNA is considered.

(ii) Methods

(1) Organism and Conditions for Growth.

E. coli B was grown in a Tris-buffered medium which contained, in one liter: NH₄Cl, 2 g; NaCl, 3 g; MgCl₂, 10 mg; Na₂SO₄, 26 mg; Tris-HCl (pH 7.2), 12.1 g; K₂HPO₄, 1.7 g; glucose, 5 g; and 100 mg of each of the standard amino acids except methionine. The amino acids, glucose, and phosphate were separately sterilized before addition to the remainder of the medium (Roberts et al., 1957).

To prepare (methyl-¹⁴C, ³²P)-RNA, cultures were

grown to mid-logarithmic phase, at which time, 25 ml aliquots were removed and added to an equal volume of the same sterile medium, prewarmed to 37°. The 50 ml cultures (2×10^8 cells per ml) were vigorously shaken for 45 minutes after the addition of 5 mc of sodium phosphate- ^{32}P (Charles Frosst and Co., Montreal) and 50 μc of (methyl- ^{14}C)-L-methionine (Calbiochem; Specific Activity; 10 mc/mole).

For preparation of (methyl- ^{14}C , ^{32}P)-CM-rRNA, the addition of 200 μg chloramphenicol (Parke-Davis Co.) per ml of culture medium was followed one minute later by the addition of sodium phosphate- ^{32}P (10 μc) and (methyl- ^{14}C)-L-methionine (50 μc), as indicated above.

(2) Preparation of (methyl- ^{14}C , ^{32}P)-rRNA from E. coli.

At the end of the incubation period, cultures were washed and the RNA was extracted according to the procedures outlined in Part II. After the RNA had been washed three times with each of 70% ethanol, 95% ethanol, and ether, the air-dried RNA was dissolved in two ml of water and the solution was made 2 M with respect to sodium chloride by the addition of solid salt. The solution was allowed to stand at 0° for 12 hours in order to precipitate rRNA. Following centrifugation, the precipitate was washed twice with cold 2 M sodium chloride and three times with each of 70% ethanol, 95% ethanol, and

ether. The final air-dried powder was stored at -20° .

(3) Alkali Hydrolysis of (methyl- ^{14}C , ^{32}P)-rRNA and Fractionation of the Hydrolyzates.

The (methyl- ^{14}C , ^{32}P)-rRNA and (methyl- ^{14}C , ^{32}P)-CM-rRNA (10-15 mg) were each dissolved in 400 μl of water and made 1 M with respect to alkali by the addition of 100 μl of 5 M sodium hydroxide. In this case, hydrolysis was allowed to proceed for 90 hours at 25.0° .

Radioactive hydrolyzates were supplemented with 2 μmoles of carrier alkali-stable dinucleotides prepared from wheat embryo rRNA (Part II), and the hydrolyzates were resolved into N, Np, NxpNp, and pNp fractions by step-wise elution from DEAE-cellulose, according to the procedures detailed in Part II of the thesis.

(4) Paper Chromatographic Resolution of (methyl- ^{14}C , ^{32}P)-dinucleoside Phosphates).

Following the conversion of alkali-stable dinucleotides to dinucleoside phosphates, two-dimensional chromatography was used to resolve these compounds (Part I). Individual dinucleoside phosphates were located by their ultraviolet quenching on paper chromatograms, excised, and counted directly in scintillation fluid (Bray, 1960; see Part II). ^{14}C and ^{32}P were counted simultaneously in a Nuclear Chicago scintillation counter, and corrections were made for the overlap of ^{32}P counts in the ^{14}C channel (23%). Where the radioactive dinucleoside phos-

phates were required for further characterization, it was found to be more convenient to elute the excised areas in water and count a small aliquot while conserving the remainder of the eluate for characterization studies. This eliminated the need to recover the compounds from paper strips which had been exposed to scintillation fluid (Part II).

(5) Characterization of Individual (methyl-¹⁴C,
³²P)-Dinucleoside Phosphates.

After elution from paper chromatograms, the dinucleoside phosphates were individually desalted, and then hydrolyzed by purified snake venom phosphodiesterase in order to produce equimolar amounts of the constituent 2'-O-methyl nucleoside and normal 5'-nucleotide. The procedure is described in Part I. The products of digestion were resolved by one-dimensional paper chromatography with standard marker compounds.

(6) Procedures Used for Snake Venom-Induced Digestion of (methyl-¹⁴C)-rRNA, and for Resolution of the Resulting Hydrolysis Products.

(Methyl-¹⁴C)-rRNA was hydrolyzed to its constituent nucleosides and 2'-O-methyl nucleoside-5'-phosphates by Russell viper venom. 10 to 15 mg of RNA was dissolved in 1250 μ l of water and mixed with 250 μ l of 1 M ammonium formate buffer, pH 9.2, and 500 μ l of whole venom (2 mg/ml). Incubation was allowed to proceed for 24 hours at 37^o.

After diluting the hydrolyzate to about 50 ml with water, the pH was adjusted to neutrality and the solution was made up to a final volume of 250 ml.

Resolution of the N and pNx compounds was accomplished by chromatography on a 2.5 cm x 10 cm DEAE-cellulose column (formate-form). The hydrolyzate was applied to the column and all traces of nucleosides were removed by copious washing of the column with water. The pNx compounds were removed from the column with 100 ml of 1 M pyridinium formate, pH 4.5. The pyridine solution was evaporated to dryness and re-evaporated from 0.5 M ammonium hydroxide solution to convert the pyridinium salts to ammonium salts.

The final residue was dissolved in 200 μ l of water, containing marker pNx compounds, and then chromatographed in the one-dimensional system. The marker pNx solution contained about 0.1 μ mole of each of pCx, pGx, pAx, and pUx, and had been prepared from wheat embryo rRNA according to the procedure outlined in Part I of the thesis.

Areas containing marker pNx compounds, as well as all remaining areas of the chromatogram, were excised, and eluted with water. Small aliquots of the eluent from each area of the chromatogram were counted.

The (methyl-¹⁴C)-pN⁴MeCx component was desalted and further characterized by electrophoresis and paper

TABLE XII

The Distribution of methyl-¹⁴C among the Dinucleoside Phosphates Prepared from E. coli (methyl-¹⁴C)-rRNA

Dinucleoside Phosphate*	<u>methyl</u> - ¹⁴ C ^a (c.p.m.)	Mole % of ^a the Dinuc- leotides	<u>methyl</u> - ¹⁴ C ^b (c.p.m.)	Mole % of ^b the Dinuc- leotides
AxpA	224	3.1	261	5.6
AxpG-GxpA	87	1.2	74	1.5
AxpC	47	0.6	34	0.7
AxpU	56	0.7	30	0.6
GxpG	1515	20.9	894 [2782]	19.2
GxpC-CxpG	29	0.4	24	0.5
GxpU	169	2.3	260	5.5
CxpA	94	1.3	51	1.1
CxpC	1493	20.5	952 [2926]	20.5
CxpU-UxpC	164	2.3	83	1.7
UxpA	18	0.2	58	1.2
UxpG	1471	20.2	1000 [3206]	21.5
UxpU	78	1.1	67	1.5
N ⁴ MeCxpC	3666	25.2**	1743 [3038]	18.8**

* Certain of the isomeric dinucleoside phosphates are not separated by the two-dimensional paper chromatographic system used in these studies, and the values for the unresolved pairs have been listed.

** The methyl-¹⁴C value in the adjacent column was halved in the case of N⁴MeCxpC.
Values in parentheses (column 4) are included for comparison purposes, and were obtained in an analysis of uniformly labeled ¹⁴C-rRNA (Part II).

a rRNA extracted from E. coli B grown in the Tris-buffered medium described in "Methods" of this section of the thesis.

b rRNA extracted from E. coli B grown in the phosphate-buffered medium (medium C) described in Part II of the thesis.

chromatography.

(iii) Results of Analyses

(1) The in vivo Incorporation of Methyl-¹⁴C of Methionine into the Sugar Constituents of *E. coli* rRNA.

The distribution of methyl-¹⁴C among the different carrier dinucleotides is presented in Table XII, and it is noteworthy that similar relative proportions were obtained when the (methyl-¹⁴C)-L-methionine was added to either the Tris-buffered culture medium (described in Methods of this section), or to the phosphate-buffered medium (medium C), described in Part II. The mononucleotides prepared from alkali hydrolyzates of RNA were chromatographed in two dimensions and examined for ¹⁴C and ³²P radioactivity. Measurements of the ¹⁴C/³²P ratios after paper chromatographic purification of the four principal (unmethylated) nucleotides in the hydrolyzates, showed that incorporation of ¹⁴C into the carbon-skeleton of nucleotides could not have exceeded 1% of the ¹⁴C-radioactivity found in the dinucleotides. The relative amounts of radioactivity in the four major alkali-stable dinucleotides recovered from alkali hydrolyzates of uniformly ¹⁴C-labeled *E. coli* rRNA (Part II) are also presented in Table XII in order to show that the N⁴MeCxpCp sequence is present in an amount similar to other sequences, but contains roughly twice as much (methyl-¹⁴C)-radioactivity.

TABLE XIII

The Distribution of methyl- ^{14}C and phosphate- ^{32}P Among
the Principal Dinucleoside Phosphates Derived from
(methyl- ^{14}C , phosphate- ^{32}P)-rRNA

Dinucleoside Phosphate	<u>methyl-^{14}C</u> (c.p.m.)	<u>phosphate-^{32}P</u> (c.p.m.)	$^{14}\text{C}/^{32}\text{P}$
GxpG	1515	2096	0.72
CxpC	1493	2067	0.72
N^4MeCxpC	3666	2234	1.64
UxpG	1471	1809	0.81
$\text{N}^6\text{N}^6\text{diMeApN}^6\text{N}^6\text{diMeA}$	345	100	3.45

(2) Characterization of N⁴,2'-O-dimethyl Cytidine by in vivo Incorporation of Methyl-¹⁴C and Phosphate-³²P into E. coli rRNA.

The structure of N⁴,2'-O-dimethyl cytidine has been deduced from evidence which is severely limited by the trace amount of the component available for examination, and it was felt that it would be useful to have confirmatory evidence to support the structural assignment. The four major dinucleotides were recovered from an alkali hydrolyzate of (methyl-¹⁴C, ³²P)-rRNA and, as expected, the N⁴,2'-O-dimethyl cytidine-containing dinucleoside phosphate was found to contain twice as much methyl-¹⁴C per unit of ³²P radioactivity as did the other three 2'-O-methyl ribose-containing dinucleoside phosphates. These data have been summarized in Table XIII, along with data on N⁶,N⁶diMeApN⁶,N⁶diMeA, which had a ¹⁴C/³²P ratio about four times greater than found for the monomethylated 2'-O-methyl ribose-containing alkali-stable dinucleoside phosphates.

Furthermore, following hydrolysis with purified venom phosphodiesterase, the ³²P from N⁴MeCxpC and CxpC migrated with cytidine 5'-phosphate, and the ¹⁴C migrated with N⁴, 2'-O-dimethyl cytidine in the N⁴MeCxpC-hydrolyzate, and with Cx in the case of the CxpC-hydrolyzate, after paper chromatographic separation of the products in system A. The paper chromatographic mobilities of the

TABLE XIV

The Distribution of methyl- ^{14}C and phosphate- ^{32}P Between Carrier 2'-O-methyl Nucleoside and Normal 5'-nucleotide After Hydrolysis of Doubly-labeled Dinucleoside Phosphates with Venom Phosphodiesterase

Dinucleoside Phosphate	<u>methyl-^{14}C</u> in the nucleoside (c.p.m.)	<u>phosphate-^{32}P</u> in the nucleotide (c.p.m.)	$^{14}\text{C}/^{32}\text{P}$
CxpC	394	565	0.69
N ⁴ MeCxpC	1220	865	1.41

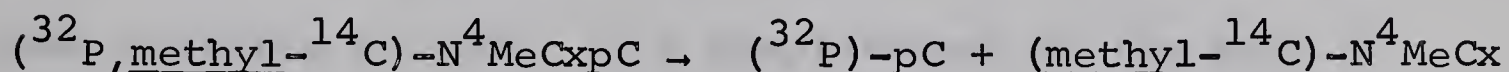
Figure 21

5 3	← ORIGINS →	2 5
3 3		4 2
4 3		5 5
pc 865 13		pc 565 15
2 2		2 3
12 12		3 4
11 9		6 9
4 3		5 9
4 11		Cx 19 394
29 36		2 4
3 13	0 3	
2 3	1 6	
4NMeCx 32 1220	0 2	
17 10	16 25	
3 7	0 3	
1 21	0 1	
1 18		
0 3		
1 2		

Legend of Figure 21

The distribution of ^{14}C - and ^{32}P -radioactivity among the carrier 2'-O-methyl nucleosides and 5'-nucleotides after one-dimensional paper chromatography (system A) of the products formed by venom phosphodiesterase hydrolysis of the doubly-labeled dinucleoside phosphates. Each of the carrier 2'-O-methyl nucleosides and 5'-nucleotides was detected by ultraviolet-quenching, excised, and placed in scintillation fluid for the simultaneous determination of both ^{14}C - and ^{32}P -radioactivity. The remaining areas of the chromatogram were excised as paper strips and counted in the same way. The hydrolysis products of N^4MeCxpC , were chromatographed at the left of the marker-nucleosides, while the hydrolysis products of CxpC were chromatographed at the right of the marker-nucleosides. The numbers at the right in each of the individual areas represent (methyl- ^{14}C)-radioactivity (c.p.m.) and the numbers at the left in each area show the ^{32}P -radioactivity (c.p.m.).

different carrier compounds have been illustrated in Part I of the thesis and the nature of the hydrolytic characterizations can be schematically illustrated as follows:



Quantitative data for the phosphodiesterase hydrolysis of doubly-labeled dinucleoside phosphates have been summarized in Table XIV, and, as expected, it was found that the ratio of ^{14}C in the nucleoside to ^{32}P in the nucleotide was twice as great in the case of N^4MeCxpC as in the case of CxpC . The chromatographic resolution of the carrier nucleosides and 5'-nucleotides in each of the two hydrolyzates, together with the distribution of both ^{14}C and ^{32}P labels is illustrated in Figure 21.

(3) The Effects of Chloramphenicol on the in vivo Incorporation of methyl- ^{14}C from Methionine into the Glycosyls of E. coli rRNA.

The (methyl- ^{14}C , ^{32}P)-rRNA and (methyl- ^{14}C , ^{32}P)-CM-rRNA used for these experiments were prepared from organisms grown under the conditions described in section III, (ii), (1). The amount of radioactive phosphate was diminished 50⁰-fold from the preceeding experiments in this section, since there was no need to obtain measurable amounts of ^{32}P in the dinucleotides. Sedimentation in a sucrose-

density gradient after the method of Mitsui, Ishihama, and Osawa (1963) showed that the ^{32}P -CM-RNA exhibited the characteristic features expected from the earlier studies by Dubin and Elkhort (1964): (i) preferential synthesis of sRNA relative to rRNA, (ii) displacement of the peak of radioactivity for the slower-sedimenting rRNA component to a position slightly ahead of the ultraviolet absorbance peak of carrier 16S RNA, and, (iii) reduction in the proportion of faster-sedimenting component in radioactive rRNA relative to the roughly 67% proportion for the faster-sedimenting component in the carrier rRNA.

^{32}P -incorporation into the four principal ribonucleotides was measured after resolution of the compounds by two-dimensional filter paper chromatography. It was found that the relative amounts of ^{32}P in Ap, Gp, Cp, and Up were nearly identical for $(^{14}\text{C}, ^{32}\text{P})$ -rRNA and $(^{14}\text{C}, ^{32}\text{P})$ -CM-rRNA, although the total radioactivity in $(^{14}\text{C}, ^{32}\text{P})$ -rRNA was about 2.5 times greater than in $(^{14}\text{C}, ^{32}\text{P})$ -CM-rRNA. Based on ultraviolet absorbance (260 m μ), the recoveries of rRNA, including carrier rRNA derived from equivalent additions of carrier cells in the two cases, were nearly identical for cultures grown in the absence and presence of chloramphenicol. The measured amounts of methyl- ^{14}C incorporated into the alkali-stable dinucleotide sequences of $(^{14}\text{C}, ^{32}\text{P})$ -CM-rRNA were multiplied by 2.5 for comparison with the

TABLE XV

The Effect of Chloramphenicol on the Incorporation of methyl-¹⁴C into the Principal Alkali-stable Dinucleotide Sequences of E. coli rRNA

Dinucleoside Phosphate	methyl- ¹⁴ C (c.p.m.)		% Inhibition
	-Chloramphenicol	+Chloramphenicol*	
GxpG	2662	603 x 2.5 = 1507	44
CxpC	2770	427 x 2.5 = 1068	61
N ⁴ MeCxpC	7319	305 x 2.5 = 769	90
UxpG	2692	103 x 2.5 = 258	90

* See text for the explanation of the factor x 2.5.

measured amounts of methyl-¹⁴C incorporated into the same amount of newly-formed (¹⁴C, ³²P)-rRNA recovered from the control cultures. The results of the experiments have been summarized in the data of Table XV, and it is apparent that there was a pronounced reduction of methyl-¹⁴C per unit of newly-synthesized rRNA following the addition of chloramphenicol to the culture medium, relative to a control culture growing normally in the absence of chloramphenicol. The reduced incorporation in the presence of chloramphenicol was more marked in the case of N⁴MeCxpCp and UxpGp than with GxpGp and CxpCp.

(4) The Isolation of (methyl-¹⁴C)-pN⁴MeCx from Whole Venom Digests of (methyl-¹⁴C)-rRNA.

Honjo, Kanai, Furukawa, Mizuno, and Sanno (1964) first noted the resistance of the 2'-O-methyl nucleoside 5'-phosphates to the 5'-nucleotidase in whole venom digests of RNA. The resistance of these compounds to the 5'-nucleotidase has made it possible to isolate the pNx compounds from whole venom hydrolyzates of wheat embryo sRNA (Hudson, Gray, and Lane, 1965). The compound, pN⁴MeCx, has been isolated in quantities sufficient for its spectral characterization (Part I), and it was found that the spectra of the nucleotide were the same as those of the corresponding nucleoside, derived from the alkali-stable dinucleotide N⁴MeCxpCp. To further characterize this compound, it was isolated from whole venom digests

Figure 22

	ORIGIN 228
	133
	100
	106
	160
	239
	2 89
	296
	1350
	92 8
	161
C	pCx 7198
	604
G	pGx 2758
	500
A	pN ⁴ MeCx 14343
	890
U	pUx 9040
	248
	385
	412
	340

Legend of Figure 22

The distribution of (methyl-¹⁴C)-radioactivity among carrier 2'-O-methyl nucleoside 5'-phosphates (pNx) after one-dimensional paper chromatography (system A) of the (methyl-¹⁴C)-pNx compounds isolated from whole venom digests of E. coli (methyl-¹⁴C)-rRNA. Each 2'-O-methyl nucleotide was detected by ultraviolet-quenching, excised, and eluted in water. Small aliquots of each eluent were placed in scintillation fluid for the determination of ¹⁴C-radioactivity in a Beckman CPM-100 liquid scintillation counter. The remaining areas of the chromatogram were excised, eluted in water, and the eluents were counted in the same way. The areas of maximum radioactivity on the chromatogram, co-chromatographed precisely with carrier pNx compounds, which had been isolated from wheat embryo rRNA. Since 2'-O-methyl adenosine is not present in E. coli rRNA, no (methyl-¹⁴C)-radioactivity was detected in this area of the chromatogram. However, areas of radioactivity were detected, which corresponded to the mobility of pN⁴MeCx (see Figure 5). To simplify the figure, the position of carrier pAx has not been indicated, but the area corresponding to the mobility of pN⁴MeCx has been indicated, together with the total amount of (methyl-¹⁴C)-radioactivity detected. The number in each area of the chromatogram represents the total amount of radioactivity (c.p.m.) detected in that area.

TABLE XVI

The Distribution of methyl-¹⁴C among Carrier 2'-O-methyl Nucleosides and 2'-O-methyl Nucleoside 5'-phosphates after Isolation of pN⁴MeC \bar{x} from Whole Venom Digests of methyl-¹⁴C-rRNA

Separation Technique	<u>methyl</u> - ¹⁴ C (c.p.m.)				
	<u>pCx</u>	<u>pGx</u>	<u>pAx</u>	<u>pN⁴MeCx</u>	<u>pUx</u>
C ^a	100	50	90	6720	39
	<u>pCx+pAx+pN⁴MeCx</u>			<u>pGx</u>	<u>pUx</u>
E ^a	486			17	0
	<u>Cx</u>	<u>Gx</u>	<u>Ax</u>	<u>N⁴MeCx</u>	<u>Ux</u>
C ^b	15	0	30	2800	7
	<u>Cx + Ax + N⁴MeCx</u>			<u>Gx</u>	<u>Ux</u>
E ^b	1103			5	40

The symbol C designates one-dimensional paper chromatography in system A. The symbol E designates electrophoresis in 1 M formic acid at pH 2.0.

^a The material used in these experiments had been recovered and desalted from an initial chromatogram (see Figure 22), in which (methyl-¹⁴C)-pN⁴MeCx had been resolved from (methyl-¹⁴C)-pCx, (methyl-¹⁴C)-pGx and (methyl-¹⁴C)-pUx.

^b The material used in these experiments was prepared by recovering pN⁴MeCx from a paper chromatogram (C^a), treating it with E. coli phosphomonoesterase, and desalting the product by charcoal adsorption and elution.

of (methyl- ^{14}C)-rRNA according to the procedures outlined earlier.

The (methyl- ^{14}C)-pNx components from E. coli rRNA were co-chromatographed with marker pNx compounds from wheat embryo rRNA, and the distribution of radioactivity is presented in Figure 22. $\text{N}^4, 2'\text{-O-dimethyl cytidine } 5'\text{-phosphate}$ is not present in detectable quantities in hydrolyzates of wheat embryo rRNA, and the radioactivity presumed to correspond to (methyl- ^{14}C)- pN^4MeCx had a mobility slightly greater than that of $2'\text{-O-methyl adenosine } 5'\text{-phosphate}$, which is present in hydrolyzates of wheat embryo rRNA, but not in hydrolyzates of E. coli rRNA. This mobility for (methyl- ^{14}C)- pN^4MeCx was expected from the results obtained in Part I of the thesis, where a spectrally observable amount of pN^4MeCx was recovered from E. coli rRNA (see Figure 5).

(Methyl- ^{14}C)- pN^4MeCx was eluted from these chromatograms and desalted according to procedures already described (see Part I). The nucleotide was subjected to chromatography and electrophoresis and, following the removal of the phosphomonoester group with E. coli phosphomonoesterase, the resulting nucleoside was also subjected to chromatography and electrophoresis. The results of these experiments are presented in Table XVI. In all cases the radioactivity resolved precisely with the carrier

pN⁴MeCx or N⁴MeCx compounds, and greater than 95% of the radioactivity was located in these carrier-areas.

(iv) Discussion

Evidence presented in Part I of this thesis was interpreted to indicate that the nucleoside, N⁴MeCx, derived by hydrolysis of N⁴MeCxpC, was in fact N⁴,2'-O-dimethyl cytidine. The conclusion was consistent with the alkali-stability of the parent compound, N⁴MeCxpC. However, this interpretation of the evidence relied heavily on the view that alkali-stability was attributable to 2'-O-methylation. The interpretation could not be considered to be definitive since, for example, the presence of deoxyribosyl, rather than 2'-O-methyl ribosyl, could have been the basis for alkali-stability. The 10-20% greater radioactivity in N⁴MeCx, relative to C, derived from uniformly ¹⁴C-labeled N⁴MeCxpC, could be considered only marginal evidence of dimethylation in N⁴MeCx, because of the limited experimental accuracy of the measurements. Thus it was felt that independent evidence for the structure of the glycosyl in N⁴MeCx was desirable.

It has been shown in this part of the work, that N⁴MeCxpC, isolated from (methyl-¹⁴C, ³²P)-rRNA, contains twice as much (methyl-¹⁴C)-radioactivity per unit of ³²P-radioactivity as do the remaining monomethylated dinucleoside phosphates prepared from the same preparation of RNA.

As might have been expected from studies of heterocycle-methylation by Mandel and Borek (1963), and by Starr and Fefferman (1964), the methyl group of methionine can serve as an efficient donor for 2'-O-methylation, and for the methylation of the exocyclic nitrogen in $N^4\text{MeCx}$.

The extensive studies by Dubin and Günalp (1967) have shown that there is a variable inhibition of in vivo methyl- ^{14}C incorporation into different heterocycles of E. coli rRNA, in the presence of chloramphenicol. For example, they have observed that incorporation of the methyl groups in $N^6,N^6\text{diMeAp}$, is almost completely inhibited by the presence of chloramphenicol in the medium but incorporation of the methyl group in Tp is only partially inhibited under the same conditions. Dubin (1966) and Dubin and Günalp (1967), using putative techniques for the assessment of 2'-O-methyl ribose, have also noted that there is inhibition of in vivo 2'-O-methylation, by chloramphenicol.

The results of the present investigation show that, as with methylation of heterocycles in rRNA, there is also a variable inhibition of in vivo methyl- ^{14}C incorporation into different ribose residues in rRNA, in the presence of chloramphenicol. For example, incorporation of the methyl groups in UxpGp and $N^4\text{MeCxpCp}$ is more severely inhibited

than is incorporation of the methyl groups in either GxpGp or CxpCp. Even if the effects of chloramphenicol on individual methylations were equivalent, it might be expected that inhibition of incorporation in the case of N⁴MeCxpCp would be more pronounced than in the other cases, if the incorporation of two methyl groups should involve two separate enzymes.

The inhibition of methyl-group incorporation into the ribose of CM-rRNA suggests that this RNA, as well as other forms of precursor rRNA, might be used as substrate, in vitro, to assay for the presence of 2'-O-methylases in homologous systems.

Part IV. The in vitro Incorporation of Methyl Groups
Into the Ribose of Escherichia coli Ribosomal
Ribonucleates.

(i) Introduction

The functional and structural significance of minor nucleoside constituents of RNA is still largely unknown. However, in the last few years, a number of reports have appeared on the physical, chemical, and functional differences between "normally methylated" and "under-methylated" RNA (Borek and Srinivasan, 1966). Such studies have been made possible by the discovery of unique amino acid auxotrophs of E. coli. These "relaxed control" (RC^{relaxed}) organisms show a loss of control over RNA-synthesis in the absence of the required amino acid. The synthesis of the primary sequence of tRNA, rRNA, and to a limited extent, DNA, is uncoupled from subsequent methylation at the polymer level. Thus, growth of the auxotroph, under conditions of amino acid-starvation, produces methyl-deficient tRNA and rRNA. The rRNA, so produced, is contained in ribonucleoprotein particles ("relaxed" particles, "starvation" particles), which are believed to be normal intermediates in the biosynthesis of ribosomes.

The in vitro methylation of precursor-particles has been demonstrated using "chloramphenicol-particles" (CM-particles) (Gordon, Boman, and Isaksson, 1964),

"methionine-starvation" particles (MS-particles) (Gordon and Boman, 1964; Manor and Haselkorn, 1967), and "histidine-starvation" particles (Manor and Haselkorn, 1967a). The studies described in this part of the thesis were performed in order to determine whether methylation of glycosyls, as with methylation of heterocycles, occurs at the polymer level, and to determine if the enzymes responsible for 2'-O-methylation are associated with the precursor particles, as has been demonstrated for several methylases specific for heterocycles in rRNA (Gordon and Boman, 1964). MS-particles were used in this study, rather than CM-particles, because, in in vitro experiments, the MS-particles have been found to accept about four times more methyl-¹⁴C from (methyl-¹⁴C)-S-adenosyl-methionine than the CM-particles (Gordon, Boman, and Isaksson, 1964).

(ii) Methods

(1) Organism and Growth Conditions.

E. coli K₁₂W6 (RC^{rel.}, methionine⁻, biotin⁻, F⁺, λ⁺) was used in the studies in this section. This "relaxed-control" mutant was grown in the basal medium of Vogel and Bonner (1956). The concentrated medium contained the following ingredients in one liter: MgSO₄·7H₂O, 10 g; citric acid·H₂O, 100 g; K₂HPO₄·anhyd., 500 g; NaNH₄HPO₄·4H₂O, 175 g. Before use, the solution was

diluted fifty-fold with water and supplemented with 0.5% glucose (autoclaved separately). The final pH was 7.0.

To produce conditions for methionine starvation of E. coli K₁₂W6, the basal medium was supplemented with 10 µg d-biotin and 500 µg L-methionine per 100 ml of medium. After growth had stopped, the cultures were vigorously shaken for an additional 1.5 hours and then poured over crushed ice. After centrifugation, the pellet of cells was washed with cold 0.001 M Tris-HCl-0.0001 M magnesium chloride buffer, pH 7.3, and recentrifuged. The resulting pellet was frozen at -20°.

(2) Preparation of Methionine-Starvation Particles.

The frozen pellet of cells (about 1.5 g) was ground with 3 g of alumina and then 10 ml of 0.001 M Tris-HCl-0.0001 M magnesium chloride buffer, pH 7.3 containing 2 µg deoxyribonuclease per ml (electrophoretically purified deoxyribonuclease from Mann Research Laboratories) was added. The solution was centrifuged twice at 10,000 r.p.m., for 10 minutes, to remove alumina and cell debris (Kurland, Normura, and Watson, 1962). The crude extract was centrifuged for 3.5 hours at 40,000 r.p.m. in a Spinco Model L ultracentrifuge. The pellet of ribosomes was resuspended in 1 ml of 0.001 M Tris-HCl-0.0001 M magnesium chloride buffer, pH 7.3 ($A_{260\text{m}\mu} = 147.6$; $A_{280\text{m}\mu} = 75.0$), while the top 8 ml was removed by means of a syringe and centrifuged for 11 hours at 36,000 r.p.m. The supernatant solution

from this centrifugation was discarded and the pellet of "methionine-starvation" particles (MS-particles) was re-suspended in 1 ml of 0.001 M Tris-HCl buffer, pH 7.3

($A_{260m\mu} = 170.1$; $A_{280m\mu} = 82.5$).

(3) Conditions for in vitro Incubation of MS-Particles with (methyl- ^{14}C)-S-adenosyl-methionine.

The conditions employed for methyl-incorporation were essentially the same as those used by Gordon and Boman (1964). Each reaction mixture contained 0.1 M Tris-HCl-0.01 M magnesium chloride, pH 8.0, 1000 μ l; 1 M ammonium chloride, 20 μ l; (methyl- ^{14}C)-S-adenosylmethionine, 100 μ l (1 μ c; Tracerlab; 30 mc/mM); ribosomes or MS-particles, 300-500 μ l; water to a total volume of 2 ml. Each sample was incubated for 1 hour at 25 $^{\circ}$.

(4) Assay of in vitro (methyl- ^{14}C)-incorporation into MS-RNA.

To each reaction mixture was added an equal volume of water-saturated phenol, and the resulting suspensions were shaken in a horizontal shaker for 20 minutes. The aqueous layers were removed and precipitated with 2.5 volumes of cold 95% ethanol. After centrifugation, the pellets were washed three times with each of 70% ethanol, 95% ethanol, and ether, and air-dried. Each pellet was dissolved in 1 ml of water and then mixed with 1 ml of carrier wheat embryo rRNA (10 mg/ml). Each solution was made 2 M with respect to sodium chloride. After 24 hours

TABLE XVII

The Distribution of methyl- ^{14}C Among the Dinucleoside Phosphates Recovered from MS-RNA After Incubation of MS-particles with (methyl- ^{14}C)-S-adenosylmethionine

Dinucleoside Phosphate*	<u>methyl</u> - ^{14}C (c.p.m.)	
	MS-Particles ^a	Ribosomes ^b
AxpA	38	28
AxpG-GxpA	85	29
AxpC	21	15
AxpU	45	10
GxpG	1005	254
GxpC-CxpG	57	30
GxpU	59	15
CxpA	31	11
CxpC	1749	235
CxpU-UxpC	78	54
UxpA	9	3
UxpG	32	162
UxpU	0	1
N ⁴ MeCxpC	0	0

* Certain of the isomeric dinucleoside phosphates are not separated by the two-dimensional paper chromatographic system used for these studies, and the value for the unresolved pairs are listed.

^a Reaction mixture contained 300 μl MS-particles ($A_{260\text{m}\mu} = 51$; $A_{280\text{m}\mu} = 24.75$).

^b Reaction mixture contained 500 μl ribosomes ($A_{260\text{m}\mu} = 73.5$; $A_{280\text{m}\mu} = 37.5$).

at 0°, the resulting pellet was washed twice with cold 2 M sodium chloride solution and three times with each of 70% ethanol, 95% ethanol, and ether. The air-dried powders were dissolved in 300 μ l of water before the addition of 100 μ l of 1 M sodium hydroxide. Hydrolysis was allowed to proceed at 37° for 18 hours.

Carrier alkali-stable dinucleotides from wheat embryo (about 2 μ mole) were added to each neutralized hydrolyzate. The procedures employed for the fractionation of hydrolyzates, and the chromatographic separation and characterization of the alkali-stable dinucleoside phosphates, were identical with those described in Part II, except that, after the complete elution of the mononucleotides from DEAE-cellulose columns, the columns were washed with water and the dinucleotides were eluted with 1 M pyridinium formate buffer, pH 4.5.

(iii) Results

There are four principal alkali-stable dinucleotides in E. coli rRNA which might be expected to contain (methyl-¹⁴C)-radioactivity. However, with MS-particles, only two of these, CxpC and GxpG, were found to be methylated in vitro (Table XVII). The fact that methylation of MS-particles is possible in the absence of supernatant from the crude extract demonstrates that at least two 2'-O-methylases are associated with the precursor particle.

Figure 23

3 ← ORIGINS → 6			
2		8	
4		9	
7		10	
11		3	
10		CxpC 552	
4		6	
0		0	
2		C 8	
G 1	C	4	
5		9	
5		1	
10		4	
10	G	Cx 1314	
Gx 556	A	0	
4		7	
5		3	
	U	3	

Legend of Figure 23

The chromatographic distribution of (methyl-¹⁴C)-radioactivity among the carrier nucleosides and 2'-O-methyl nucleosides after snake venom hydrolysis of the alkali-stable dinucleoside phosphates, derived from MS-rRNA. Following the location of each of the carrier nucleosides by ultraviolet-quenching, they were excised, and, together with the remaining areas, which were cut into paper strips, placed in scintillation fluid for radioactivity measurements. The radioactivity (c.p.m.) obtained for each area is indicated by a number. The hydrolyzate of GxpG was chromatographed at the left of the marker nucleosides, and the hydrolyzate of CxpC was chromatographed at the right of the marker nucleosides. Incomplete hydrolysis was obtained in the case of CxpC, and this is the reason for the presence of CxpC following chromatography.

In the case of "mature" ribosomes, there was very little methyl-group incorporation into the ribose of rRNA, presumably because the RNA was almost fully methylated. The low level of incorporation of methyl-¹⁴C groups in the case of "mature" ribosomes may have been due to contamination of the ribosome pellet with MS-particles, since no purification of the ribosomes and MS-particles was attempted beyond the initial differential centrifugation.

The labeled dinucleoside phosphates, CxpC and GxpG, recovered by hydrolysis of the RNA from MS-particles, were further characterized by whole venom digestion. Figure 23 shows the one-dimensional paper chromatographic separations (system A) of the hydrolysis products. In each case the radioactivity co-chromatographed precisely with the carrier 2'-O-methyl nucleosides.

(iv) Discussion

The studies of Gordon and Boman (1964) demonstrated that the MS-particles would accept methyl groups, in vitro, from S-adenosylmethionine. Gordon and Boman showed that part of the methyl-¹⁴C-radioactivity could be recovered in the heterocycles of N⁶MeA and N⁶,N⁶diMeA following hydrolysis of MS-rRNA. Their conclusion that limited methylation of the heterocycle in adenosine can occur at the

polynucleotide level in the case of rRNA, can now be extended to include limited methylation of the glycosyls in guanosine and cytidine. Presuming that in vitro methyl group incorporation is a reflection of methyl-deficiency in vivo, it can be concluded, as with CM-particles, that MS-particles are deficient in 2'-O-methyl groups.

It is interesting to observe that those alkali-stable sequences that could not be significantly methylated (<10%) in vivo, in the case of CM-rRNA, are the same ones that could not be significantly methylated in vitro, in the case of MS-rRNA, ie. UxpGp and N⁴MeCxpCp. On the other hand, those alkali-stable sequences that could be significantly methylated in vivo (40-60%), in the case of CM-rRNA, could also be significantly methylated in vitro, in the case of MS-rRNA, ie., GxpGp and CxpCp. This might indicate that CM- and MS-particles are similar in the sense that they both contain the enzymes capable of effecting 2'-O-methylation of GxpGp and CxpCp sequences, but may lack the enzymes which methylate UxpGp and N⁴MeCxpCp sequences. Further studies will be required to test this interpretation, and to establish whether the failure to observe methylation of UxpGp and N⁴MeCxpCp sequences in

CM-particles and MS-particles is related to: (i) substrate deficiency, (ii) enzyme deficiency, or (iii) inadequate conditions of pH, ionic strength, etc. for in vitro methylation. If the enzymes required for methylation of UxpGp and N⁴MeCxpCp are simply not present in CM-particles or MS-particles, but substrate is present in CM-rRNA and MS-rRNA, then it should be possible to effect in vitro methylation of these sequences by treating MS-rRNA (or CM-rRNA) with normal ribosomes, or dissociation products from normal ribosomes, in vitro, in the presence of (methyl-¹⁴C)-S-adenosylmethionine.

Part V. The Terminal Groups of Ribonucleate Chains in
Each of the 16S and 23S Components of Escheri-
chia coli Ribosomal RNA

(i) Introduction

The hydrolysis of E. coli ribosomal ribonucleates was found to produce nucleosides (N) and nucleoside diphosphates (pNp) in amounts sufficient for their characterization by standardized chromatographic and spectral techniques (Lane, 1962; Lane, Diemer, and Blashko, 1963). The quantity of nucleosides was only 0.07 - 0.08 mole % of the constituent nucleotides in the RNA preparations, and did not increase with time, when the hydrolysis in 1 M sodium hydroxide solution, at room temperature, was extended from 24 to 90 hr (Lane, 1962). Since the nucleoside diphosphates can be recovered in amounts nearly equimolar with the quantity of nucleosides, it has been concluded that the ribonucleate chains had a formal structure based on a repeating 5'-mononucleotide unit, and had an average degree of polymerization in the neighborhood of 1300 residues, ie. (pN)_n, where n = 1300, before fragmentation (Lane, Diemer, and Blashko, 1963).

pNpNpNpNpNpN

3'-linked, or
5'-phosphomono-
ester terminus

5'-linked, or
3'-hydroxyl
terminus

More extensive studies led to the same general conclusions about the structure of rRNA from wheat embryo (Lane and Allen, 1961; Lane, 1965; Diemer, McLennan, and Lane, 1966). Whereas adenosine and uridine comprise about 80% of the nucleosides in alkali hydrolyzates of E. coli rRNA (Lane, 1962), and the purine nucleoside diphosphates account for most of the pNp compounds, it has been found that there are similar amounts of adenosine, guanosine, cytidine, and uridine among the nucleosides in alkali hydrolyzates of wheat embryo rRNA, and, further, that the purine nucleoside diphosphates do not account for more than 50% of the pNp compounds, in the case of wheat embryo rRNA (Lane, 1965). These compositional differences between the microbial and plant rRNA terminals stand in sharp contrast with the uniformity found for animal, plant, and microbial sRNA-terminals, where it has been found that adenosine and guanosine 2'-(3'), 5'-diphosphate account for about 80%, or more, of the nucleosides and nucleoside diphosphates, respectively, in alkali hydrolyzates (Dunn, 1959; Starr and Goldthwait, 1960; Herbert and Canellakis, 1960; Bell, Tomlinson, and Tener, 1963; Hudson, Gray, and Lane, 1965).

The earlier studies of rRNA were performed with gram quantities of RNA, in order to obtain sufficient amounts of nucleosides and nucleoside diphosphates,

for spectral characterization, after isolation from alkali hydrolyzates, by standardized chromatographic techniques. It was not feasible to prepare the individual 16S and 23S components of E. coli rRNA, or the individual 18S and 28S components of wheat embryo rRNA in gram-quantities for examination of chain terminals. However, in the case of E. coli, it is possible to prepare uniformly ^{14}C -labeled rRNA of sufficiently high specific activity for end group analysis, and to effect the separation of the labeled 16S and 23S components on a milligram-scale. Thus, uniformly ^{14}C -labeled rRNA prepared from E. coli was resolved into 16S and 23S components by sucrose density gradient centrifugation, and the chain termini have been characterized with the aid of carrier nucleosides and nucleoside diphosphates added to the neutralized alkali hydrolyzates of the individual ^{14}C -labeled 16S and 23S components.

(ii) Methods

(1) Preparation and Characterization of Ribonucleates.

The preparation and characterization of uniformly ^{14}C -labeled 16S and 23S ribonucleates was described in Part II.

(2) Analysis of Alkali Hydrolyzates of the Ribonucleates.

The ribonucleates were hydrolyzed in 1 M sodium hydroxide solution at $25.0 \pm 0.05^\circ$ for 24 or

96 hr at a ribonucleate concentration of approximately 1 mg/100 μ l of hydrolyzate. The hydrolyzate was neutralized, and carrier nucleosides (0.25 μ mole of each of adenosine, guanosine, cytidine, and uridine), nucleoside diphosphates (0.25 μ mole of each of pAp, pGp, pCp, and pUp), and alkali-stable dinucleotides (2 μ moles total, in the proportions found in wheat embryo rRNA) were added. The hydrolyzate was then diluted to 250 ml with water, and fractionated on a DEAE-cellulose column, as described earlier (Part II), using urea-containing eluents according to the method of Tomlinson and Tener (1963).

The column effluent containing nucleosides, was adjusted to pH 4.5, and passed through a 100 mg disc of acid-washed Norit charcoal (Lane and Butler, 1959), mounted between two 1 in. layers of No. 545 Celite in a chromatographic tube 2.5 cm in diameter. After quantitative adsorption of the nucleosides to charcoal, and removal of residual salt by three 15 ml water washes of the charcoal-Celite column, the nucleosides were eluted with 100 ml of 10% pyridine in 50% aqueous ethanol. The effluent containing nucleosides was evaporated to dryness in a flash evaporator at 40^o, and the dry residue was recovered in two 4 ml aliquots of water, and again evaporated to dryness in an evapomix. The dry

residue was dissolved in 100 μ l of water and banded on three separate strips of paper for electrophoresis, using 0.025 M sodium tetraborate buffer, pH 9.2, in a Durrum-type paper electrophoresis cell (Model R, Series D, Beckman). After 90 min at 500 V, the nucleosides were detected by their ultraviolet absorbance, eluted in 100 ml of water, and, after the pH of the eluent was adjusted to 4.5, the nucleosides were desalted and evaporated to dryness as described above. The dry residue of nucleosides was dissolved in 100 μ l of water and spotted for paper chromatographic resolution of the four ribonucleosides using system A. The recovery of carrier nucleosides from hydrolyzates, after paper chromatography and elution in 0.1 M hydrochloric acid solution, was usually 70-80%.

The nucleoside diphosphates were prepared from different sources to provide carriers for end group analyses. The pAp (adenosine 2'(3'),5'-diphosphate) was prepared by digestion of TPN and CoA with whole snake venom as described by Wang et al. (1954). The pGp (guanosine 2'(3'),5'-diphosphate) was prepared from large-scale alkali hydrolyzates of wheat embryo sRNA (Hudson, Gray, and Lane, 1965). The pCp and pUp (cytidine 2'(3'),5'-diphosphate and uridine 2'(3'),5'-diphosphate) were prepared in 15% yield by the concerted action

of pancreas ribonuclease and snake venom enzymes on wheat embryo rRNA (Crestfield and Allen, 1956). For this purpose, wheat embryo rRNA (500 mg), Russell viper venom (8 mg), and pancreas ribonuclease (1 μ g), were incubated together in a total volume of 100 ml of 0.25 M ammonium formate buffer, pH 9.2, at 37^o for 24 hr, and then the pC>p and pU>p compounds were recovered by fractionation of the hydrolyzate on DEAE-cellulose. The pC>p and pU>p compounds were converted to pCp and pUp, respectively, by incubation in 1 M sodium hydroxide solution for 1 hr at room temperature.

The recovery of carrier pNp compounds from alkali hydrolyzates was 50-80% when the final elution of two-dimensional chromatograms was performed with 5 ml of 0.1 M hydrochloric acid solution for each of the pNp compounds. As noted earlier, it is essential to employ large DEAE-cellulose columns in order to effect a nearly quantitative recovery of the pNp compounds (Lane, 1965), but the ease of working with small columns dictated the acceptance of lower recoveries in the present studies.

(3) Radioactivity Measurements.

Nucleosides and nucleoside diphosphates were detected on paper chromatograms, and spectrally characterized, according to procedures described in Parts I and II. Aliquots (1 ml) of chromatographic eluents were

added to 10 ml of Bray's solution (Bray, 1960) and counted in a Nuclear Chicago scintillation counter (see Part II). Background radioactivity was measured for each vial, containing scintillation fluid, before the addition of experimental samples. A 10 min counting period was used for all measurements in the scintillation counter. The counting efficiency of the acid eluents was found to be linear between 10 and 10^5 c.p.m., and the counting of acid eluents was 60-70% as efficient as when the same amount of radioactivity was added in 25 μ l of water to the scintillation fluid. Nucleosides and nucleotides were counted with equal efficiency when added to vials in 0.1 M hydrochloric acid solution. The total radioactivity in the hydrolyzates from which the nucleosides and nucleoside diphosphates were recovered, was measured under the same conditions of counting efficiency as used for the nucleosides and nucleoside diphosphates: a 100 μ l aliquot of the 250 ml hydrolyzate was counted after the addition of 1 ml of the acid eluent from a paper chromatographic blank to the scintillation vial.

(iii) Methodology

It was found that 96-98% of the total radioactivity applied to chromatograms was confined to the nucleoside carrier areas following one-dimensional

paper chromatography. There was never any significant radioactivity between, behind, or ahead of the nucleosides. It was absolutely imperative to employ the borate-electrophoresis step for preparation of nucleosides for analysis. Significant amounts of bases were always present, together with nucleosides, in the alkali hydrolyzates, and the bases were separated from the nucleosides by the borate electrophoresis step. The quantity of bases tended to be spurious in different hydrolyzates, unlike the quantity of nucleosides, which was remarkably constant for different hydrolyzates and for different times of hydrolysis in 1 M sodium hydroxide solution. The quantity of bases after 24 hr was roughly one-third as great as the quantity of nucleosides. The quantity of bases after 96 hr in 1 M alkali exceeded the quantity of nucleosides, and it is thought that the bases are produced by a trace of N-glycosyl cleavage of the nucleotides.

Only about 50% of the radioactivity applied to chromatograms in the case of uniformly labeled-¹⁴C experiments was confined to the carrier pNp compounds following two-dimensional paper chromatography. Sectioning of the entire chromatogram indicated that the remaining 50% of the radioactivity was broadly distributed over the chromatogram. The radioactivity in the

pAp and pGp areas was about 10 times greater than that found in surrounding areas of comparable size. Because of this background of radioactivity in pNp analyses, the analytical figures for the nucleosides are far superior for purposes of chain length estimates. In some cases, the pNp compounds were eluted from two-dimensional paper chromatograms, desalted, and then converted to nucleosides by treatment with E. coli phosphomonoesterase (see Part I). The resulting nucleosides were passed through a DEAE-cellulose column, before resolution by two-dimensional paper chromatography. After this procedure, about 80% of the radioactivity was confined to nucleoside areas of the chromatogram, and the relative proportions of the different nucleosides were in agreement with the proportions found for the corresponding nucleoside diphosphates by direct analysis.

The occurrence of the four nucleoside diphosphates, including small amounts of pCp and pUp, has been independently shown by chromatographic and spectral characterization of the compounds after alkali hydrolysis of gram quantities of ribosomal ribonucleates from E. coli.

It is noteworthy that the alkali-stable trinucleotides found in wheat embryo rRNA were virtually

TABLE XVIII

Effect of Hydrolysis Time on the Quantity of Nucleosides Formed When a Preparation of 23S RNA was Incubated in 1 M Sodium Hydroxide Solution at 25.0°

Time of Hydrolysis (h)	Nucleoside	Carrier Recovery (%)	Measured Radioactivity (c.p.m.)	Radioactivity Corrected for Recovery (c.p.m.)	Mole % of Total Nucleotides	Mole % of Total Nucleosides
24*	A	85	268	315	0.009	19
	G	73	96	131	0.004	9
	C	85	56	66	0.002	4
	U	80	856	1070	0.032	68
96†	A	85	292	344	0.010	20
	G	64	80	125	0.004	7
	C	82	112	136	0.004	9
	U	80	784	980	0.033	64

* The total radioactivity in the hydrolyzate from which the nucleosides were recovered was 3.5×10^6 c.p.m. Since the hydrolyzate contained 58% purine nucleotides and 42% pyrimidine nucleotides, by analysis, it was possible to relate the moles of nucleoside to moles of nucleotide, using weighted relative specific activities for the purine nucleotides (and nucleosides) and pyrimidine nucleotides (and nucleosides). Thus, the weighted relative specific activities were: 0.96 for the nucleotides in the total hydrolyzate, 0.90 for the pyrimidine nucleosides, and 1.0 for the purine nucleosides. The measured specific activities for the pyrimidine nucleotides were, as expected for uniform ^{14}C -labeling, 0.9 times as great as the specific activities for purine nucleotides. An example of the calculation used to arrive at the figures in the second last column of the table follows for uridine of the 24-h hydrolyzate: the mole % uridine is $(100 \times 1070/0.9)/(3.5 \times 10^6/0.96) = 0.032$.

† The total radioactivity in the hydrolyzate from which the nucleosides were recovered was 3.19×10^6 c.p.m.

TABLE XIX

Summary of Data from Quantitative Analyses of Several 16S and 23S RNA Preparations for Nucleosides and Nucleoside Diphosphates Formed by Alkali Hydrolysis

Ribonucleate Specimen	A	G	C	U	Total Nucleosides	pAp	pGp	pCp	pUp	Total Nucleoside Diphosphates
Mole per 100 moles of total nucleotides										
16S RNA	0.047 +0.003	0.008 +0.004	0.008 -0.002	0.015 +0.003	0.078 +0.007	0.044 +0.007	0.027 +0.015	0.011 +0.006	0.025 +0.005	0.11 +0.026
23S RNA	0.009 +0.000	0.003 +0.001	0.002 +0.000	0.033 +0.001	0.047 +0.002	0.011 +0.002	0.033 +0.003	0.005 +0.002	0.007 +0.003	0.056 +0.009
Mole % of total nucleosides or nucleoside diphosphates										
16S RNA	60	10	10	19		41	25	10	23	
23S RNA	19	6	4	70		20	59	9	12	

The data for 16S RNA summarize the results obtained in six independent analyses and the data for 23S RNA summarize the data obtained in three independent analyses. The values for components found in quantities less than 0.01 mole % of the total nucleotides have been quoted to only one significant figure because of the reduced analytical precision of such measurements. The reason for the greater standard deviation from the mean value in the case of the nucleoside diphosphates has been explained under "Methodology" of this section of the thesis. The values in the last two rows of the table do not sum to 100% because the figures have been rounded off to two figures.

absent from E. coli rRNA. For this reason, the paper chromatographic resolution of the nucleoside diphosphates was not complicated by the presence of trinucleotides, which would, if present, be eluted together with pNp compounds from DEAE-cellulose columns.

(iv) Results

The data of Table XVIII illustrate that the quantity of nucleosides in an alkali hydrolyzate of 23S RNA does not increase when the time of hydrolysis in 1 M sodium hydroxide solution at 25.0° is increased from 24 to 96 hr. The primary data of Table XVIII also show, in a more detailed fashion, the type of information obtained in the individual experiments contributing to the summary presented in Table XIX. The results of several end-group analyses of the individual 16S and 23S preparations are shown in Table XIX, and it is immediately apparent that the principal 5'-linked terminus in 16S RNA, is adenosine, and the principal 5'-linked terminus in 23S RNA, is uridine. The data for nucleosides can be utilized to calculate mean chain lengths of $100/0.078 = 1.3 \times 10^3$ nucleotide residues for 16S RNA and $100/0.047 = 2.1 \times 10^3$ nucleotide residues for 23S RNA. As mentioned earlier in the Methodology section, only the data for 5'-linked termini can be considered sufficiently reliable for purposes

TABLE XX

Quantitative Results Obtained for Two Particular Preparations of 16S and 23S E. coli rRNA

Ribonucleate Specimen	A	G	C	U	Total Nucleosides	pAp	pGp	pCp	pUp	Total Nucleoside Diphosphates
Mole per 100 moles of total nucleotides										
16S RNA	0.047	0.004	0.007	0.014	0.072	0.041	0.017	0.007	0.019	0.084
23S RNA	0.017	0.012	0.011	0.045	0.085	0.009	0.033	0.006	0.007	0.055
Mole % of total nucleoside or nucleoside diphosphates										
16S RNA	65	6	10	19		49	20	8	23	
23S RNA	20	14	13	53		17	60	11	12	

For these particular analyses, the background radioactivity interfering with nucleoside diphosphate measurements was signally low, although the 23S preparation used for the analysis showed evidence of greater polydispersity than usual. See text for further discussion.

of chain length calculations.

Because of the spurious background of radioactivity on chromatograms of the pNp compounds, certain analyses in which the background of radioactivity was much reduced, should be given more weight than other analyses in which there was a higher background of radioactivity. The values in Table XX illustrate the data obtained in two particular analyses in which the background of radioactivity, surrounding the pNp compounds, was signally low. The data of Table XX also show that the nucleosides formed by alkali hydrolysis of this particular 23S RNA preparation were much higher than were obtained for the other three preparations of 23S RNA. In fact, the net quantity of nucleosides found after alkali hydrolysis of the 23S RNA preparation of Table XX was similar to that obtained for 16S RNA. It is noteworthy that the sedimentation boundary for the 23S RNA preparation, which yielded a high proportion of nucleosides, was decidedly broader than that illustrated in Figure 16 for the other 23S RNA preparations.

(v) Discussion

It is interesting to note that the slow-sedimenting component of E. coli rRNA is rich in 5'-linked adenosine terminals since Hunt (1965) has previously observed, using an end group labeling technique, that the slower-

sedimenting component of rabbit reticulocyte rRNA is also rich in terminal 5'-linked adenosine. Further, the fast-sedimenting component of E. coli rRNA is rich in 5'-linked uridine termini, as Hunt has also found to be the case with the 5'-linked termini in the fast-sedimenting component of rabbit reticulocyte rRNA. It will be of interest to survey the corresponding 5'-linked termini in the rRNA of other sources, to learn whether this correlation has general application; although it should be noted in this respect, that wheat embryo rRNA is known to have approximately 50% of its 5'-linked termini in the form of guanosine and cytidine (Lane and Allen, 1961; Lane, 1965; Lee and Gilham, 1965). McIlreavy and Midgley (1967) have recently applied the end group labeling technique (Hunt, 1965) to the individual 16S and 23S components of E. coli rRNA, and have also noted a preponderance of 5'-linked adenosine, in 16S RNA, and a preponderance of 5'-linked uridine, in 23S RNA.

The occurrence of small amounts of 5'-linked guanosine and cytidine termini in E. coli rRNA might be indicative of terminal heterogeneity of the native rRNA chains of E. coli. It is also possible, however, that 5'-linked guanosine and cytidine terminals arise from chains which have either lost their "native" adenosine and uridine terminals, or to which the adenosine and

uridine terminals had not been added at the time the RNA was isolated from the cells. It would also seem possible, that the 16S and 23S preparations may contain a small amount of RNA which is not strictly rRNA (for example, mRNA), and that this additional RNA gives rise to the small quantities of 5'-linked guanosine and cytidine in the preparations analyzed. Questions concerning species heterogeneity within rRNA preparations are difficult to resolve by the techniques presently available for nucleate fractionation, but it is nonetheless clear that the small amounts of guanosine and cytidine found in alkali hydrolyzates are not artefacts, produced by dephosphorylation during alkali hydrolysis, since these nucleosides did not significantly increase when hydrolysis of a 16S RNA preparation was extended from 24 to 96 hr.

The presence of small quantities of other types of chain termini is frequently overlooked when a particular type of terminus is present in an amount much higher than that found for other types of termini. It should be noted, therefore, that there is almost a quantitative equivalence between the major 5'-linked terminus, uridine, and the principal 3'-linked terminus, guanosine, in the case of 23S RNA. Each of these two principal end groups is present in an amount (about 0.033 mole % of the total nucleotides) corresponding to 1 residue per 3000, which is the chain length expected from physi-

co-chemical measurements of weight average molecular weight for 23S RNA (Littauer and Eisenberg, 1959; Kurland, 1960; Stanley and Bock, 1965).

In accord with the results of studies of the in vitro enzymic synthesis of RNA by a DNA-directed polymerase from E. coli (Maitra and Nakata, 1966), where it has been found that ATP and GTP are the principal precursors of the 3'-linked termini in the synthetic product, it was found in this present study, that naturally-occurring E. coli rRNA has a preponderance of purine bases at 3'-linked termini. However, unlike the primary product formed by in vitro enzymic synthesis, the 3'-linked termini of E. coli rRNA, were released as nucleoside diphosphates (pNp), rather than as nucleoside tetraphosphates (pppNp), as has been found in the case of the product made in vitro (Maitra et al., 1965; Bremer et al., 1965).

A completely different approach for measuring 3'-linked termini in 16S and 23S RNA from E. coli has been utilized by Takanami (1967). In the case of 23S RNA, the proportions of different 3'-linked termini, reported by Takanami, show excellent correspondence with the results of this present investigation. In the case of 16S RNA, Takanami has, in accord with the results in this section of the thesis, observed greater variability, but, as in the present study, the principal 3'-linked terminal nucleo-

side constituent of 16S RNA was found to be adenosine.

Earlier analysis of gram quantities of unfract ionated E. coli rRNA (Lane, 1962), had shown a 1.5-fold excess of adenosine over uridine in alkali hydrolyzates, whereas equimolar amounts of 16S and 23S RNA would have been expected to yield more nearly equimolar amounts of adenosine and uridine on the basis of the data presented in this section of the thesis. It is now known that the large-scale preparations analyzed earlier were enriched in the N⁴MeCxpCp sequence (see Part II), characteristic of 16S RNA and, thus, the excess of adenosine over uridine was related to an enrichment of the preparations with the 16S component of rRNA. The disproportionately greater recovery of the 16S RNA might be invoked to account for the fact that the mean chain length obtained for bulk rRNA in the earlier study (Lane, 1962) was similar to that obtained for 16S RNA in the present study; however, this would not explain a similar finding with wheat embryo rRNA where preparations containing equal amounts of the fast- and slow-sedimenting components were found to have a mean number average molecular weight, by end-group analysis, similar to the weight average molecular weight expected for the slow-sedimenting component. It was pointed out earlier (Lane, 1965) that this latter observation with

wheat embryo rRNA, could reflect a degree of polydispersity, which is greater than is generally attributed to rRNA. In this event, the number average molecular weight, by end group analysis, would be expected to be lower than the weight average molecular weight, obtained by physico-chemical techniques. Material recovered from between the slow- and fast-sedimenting components of rat liver rRNA (Hastings et al., 1966) has been shown to persist at a sedimentation rate intermediate between the fast and slow components upon recentrifugation and, if such material were present in wheat embryo rRNA preparation,^s it could introduce a degree of polydispersity, sufficient to lead to a mean number average molecular weight for the bulk rRNA similar to the weight average molecular weight of the slow component.

Other explanations (Midgley, 1965; Moller and Boedtker, 1961; Appelbaum et al., 1966; Hastings and Kirby, 1966) could be advanced to account for the number average molecular weight of bulk rRNA approaching values similar to the weight average molecular weight of the slow component. It is, however, problematical, in view of the $\pm 10\%$ deviation found for single measurements, whether it is worthwhile, at this time, to attempt to

further rationalize the quantitative data, when the mean number average chain length for the 16S + 23S components (1.7×10^3), is of a similar order of magnitude to the number average chain length of the 16S component (1.3×10^3).

SUMMARY

1. The four principal alkali-stable dinucleotides present in alkali hydrolyzates of E. coli rRNA have been isolated and identified by standardized hydrolytic, chromatographic, and spectral techniques. These dinucleotides have been identified as GxpGp, CxpCp, UxpGp, and N^4 MeCxpCp, and their component 2'-O-methyl nucleosides, have been shown to account, cumulatively, for only 0.1 mole % of the constituent nucleotides in E. coli rRNA.
2. The novel component, N^4 MeCx, is the first component of a naturally-occurring polynucleotide that has been found to have methyl substituents in both the heterocycle and glycosyl parts of the same nucleoside. Evidence for N^4 -methyl substitution in N^4 , 2'-O-dimethyl cytidine has been obtained by comparison of the ultraviolet spectral properties of N^4 MeCx with those of authentic N^4 MeC at different pH values, and also by spectral comparison of the photohydrate of N^4 , 2'-O-dimethyl cytidine with the photohydrate formed from N^4 MeC. Evidence for methyl-substitution of the glycosyl in N^4 MeCx came from chemical, chromatographic, and electrophoretic studies. The structural assignment for N^4 , 2'-O-dimethyl cytidine was confirmed

by isolation of the dinucleotides from double-isotope labeled rRNA, in which case, it was shown that N⁴Me-CxpCp contained twice as much methyl-¹⁴C radioactivity per unit of ³²P, as did the three remaining monomethyl-substituted alkali-stable dinucleotides.

3. In addition to the dinucleotides which are completely stable in molar alkali at room temperature, by virtue of 2'-O-methylation, one other dinucleotide, N⁶,N⁶di-MeApN⁶,N⁶diMeAp, has been isolated from 90 hour hydrolyzates of E. coli rRNA. This sequence does not contain 2'-O-methyl ribose and is present after 90 hours in molar alkali by virtue of an extremely slow rate of hydrolysis. Identification of the dinucleotide was based on chromatographic, electrophoretic, and spectral data as well as on the isolation of the dinucleotide from doubly-labeled rRNA, where the methyl-¹⁴C radioactivity per unit of ³²P was about four times as great as in the monomethylated alkali-stable dinucleotides from the same hydrolyzate.
4. The principal alkali-stable dinucleotide sequences in E. coli sRNA were identified as GxpGp, UxpUp, CxpUp, and their component 2'-O-methyl nucleosides, were found to account, cumulatively, for 0.3 mole % of the constituent nucleotides in E. coli sRNA.

5. The pseudouridylate contents of the different E. coli RNA preparations were found to be: sRNA, 1.3 mole %; rRNA, 0.15 mole %; 16S RNA, 0.06 mole %; 23S RNA, 0.15 mole %.
6. Separation and analysis of each of the uniformly ^{14}C -labeled 16S and 23S components of E. coli rRNA, revealed that the 23S component contained three principal alkali-stable dinucleotide sequences, GxpGp, CxpCp, and UxpGp, whereas the 16S RNA component contained only one major alkali-stable dinucleotide sequence, $\text{N}^4\text{MeCxpCp}$.
7. Studies of the in vivo methylation of E. coli rRNA revealed that the methyl group of methionine could serve as a methyl-donor for 2'-O-methylation of ribose, as well as for N^4 -methylation of the exocyclic nitrogen of cytosine. The four principal alkali-stable dinucleotide sequences, which had been spectrally detected and characterized in large-scale preparations of rRNA, were the only sequences found to contain significant methyl- ^{14}C radioactivity.
8. Ribosomal precursor RNA synthesized by E. coli in the presence of chloramphenicol (CM-rRNA) showed a reduced incorporation of label from (methyl- ^{14}C)-L-methionine, into the 2'-O-methyl ribose-containing sequences. The

reduction was not uniform among the four sequences.

Methyl-¹⁴C incorporation was most severely inhibited in the case of UxpGp and N⁴MeCxpCp, but less severely inhibited in the case of GxpGp and CxpCp.

9. The activities of two 2'-O-methylases could be demonstrated in vitro by employing methyl-deficient, "methionine-starvation" particles as a source of enzyme and substrate, together with a methyl donor, (methyl-¹⁴C)-S-adenosylmethionine. Methyl-¹⁴C groups were found in the alkali-stable dinucleotides GxpGp and CxpCp.
10. The chain termini in each of the 16S and 23S components of E. coli rRNA, were analyzed following hydrolysis of the RNA in alkali. The principal 5'-linked end group of 16S RNA was found to be adenosine, and the principal 5'-linked end group of 23S RNA was found to be uridine. The principal 3'-linked end group of 16S RNA was also found to be adenosine, whereas the principal 3'-linked end group of 23S RNA was found to be guanosine. Quantitative estimates of chain length, based on analyses for 5'-linked terminals, indicated that the mean chain length for 16S RNA was about 1.3×10^3 nucleotide residues, and the mean chain length for 23S RNA was about 2.1×10^3 nucleotide residues.

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